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

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ABSTRACT: The collagenolytic serine protease (crab protease) isolated from the hepatopancreas of the fiddler crab, *Uca pugilator*, has been investigated with respect to its peptide bond specificity and catalytic properties by using noncollagenous substrates. In contrast to vertebrate collagenases, crab protease is a good general protease capable of degrading a variety of polypeptide and synthetic low molecular weight substrates. Crab protease displays a broad range of specificity, cleaving on the carboxyl-terminal side of residues with both positively and negatively charged side chains as well as hydrophobic side chains. The enzyme appears to favor tyrosyl, phenylalanyl, leucyl, and perhaps lysyl residues and, to a lesser extent, arginyl and glutamyl residues. The rate of cleavage of polypeptide substrates is similar to chymotrypsin but is significantly less than trypsin or chymotrypsin for low molecular weight esterase and amidase substrates. Crab protease is effectively inhibited by chymostatin but not by leupeptin or elastatinal. Several common chloromethyl ketone derivatives of phenylalanine and lysine are also ineffective, although crab protease efficiently cleaves at these residues in polypeptide substrates.

The collagenolytic protease isolated from the hepatopancreas of the fiddler crab, *Uca pugilator* (Eisen & Jeffrey, 1969; Eisen et al., 1970, 1973), has been shown by sequence analysis to be a serine protease (Grant et al., 1980) homologous to the pancreatic serine proteases of vertebrates. The first indication that this may be the case came from the observations that this enzyme possessed intrinsic trypsin- and chymotrypsin-like activity toward synthetic substrates and was inhibited by diisopropyl fluorophosphate and soybean trypsin inhibitor (Eisen et al., 1973). In addition to these activities, the enzyme had been shown to possess collagenolytic activity in that it cleaved native collagen under physiological conditions of pH, temperature, and ionic strength (Eisen & Jeffrey, 1969; Eisen et al., 1973). This was characterized by the cleavage of the native collagen helix at several sites in the area of the TC_{75}^{A} locus without loss of helical content. In addition, a marked decrease in the original intramolecular cross-linked β component of collagen with a corresponding increase in the monomeric α chains was also observed indicating an additional site of cleavage at the nonhelical ends of the molecule.

Since the fiddler crab is a scavenger that feeds on animal tissues frequently containing collagen, the presence of a collagenase in the hepatopancreas of these animals was not unexpected. However, most collagenases so far examined have been found to be metal-requiring neutral proteases (Seltzer et al., 1977). The crab protease was the first example of a serine protease that possessed significant collagenolytic activity. More recently, collagenolytic enzymes present in dog pancreas (Takahashi & Seifter, 1974), the fungus *Entomophthora coronata* (Hurion et al., 1979), and the insect *Hypoderma lineatum* (Lecroisey et al., 1979) have been reported which may also be serine proteases.

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In the present study, the collagenolytic protease from *U. pugilator* (crab protease) has been investigated with respect to its peptide bond specificity and catalytic properties by using noncollagenous substrates.

Materials and Methods

Crab protease was obtained from live fiddler crabs as previously described (Eisen et al., 1973). *N*-Benzoyl-L-tyrosine *p*-nitroanilide (BzTyrNA), *N*-benzoyl-L-valylglycyl-L-arginine *p*-nitroanilide (BzValGlyArgNA), *N*-benzoyl-L-tyrosine ethyl ester (BzTyrEE), L-1-(tosylamido)-2-phenylethyl chloromethyl ketone (TosPheCH_2Cl), *N*-α-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLysCH_2Cl), dansyl chloride, elastase, oxidized bovine insulin B chain, and ribonuclease A were purchased from Sigma Chemical Co. *N*-α-Benzoyl-L-arginine *p*-nitroanilide hydrochloride (BzArgNA), *N*-acetyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide (AcAlaAlaAlaNA), *N*-α-tosyl-L-leucine chloromethyl ketone (TLeuCH_2Cl), *N*-acetyl-L-alanyl-L-phenylalanine chloromethyl ketone (AcAlaPheCH_2Cl), *N*-α-tosyl-L-arginine methyl ester hydrochloride (TArgME), and *N*-benzyloxycarbonyl-1-phenylalanine chloromethyl ketone (ZPheCH_2Cl) were from Vega Biochemicals. Leupeptin and Dnp-Pro-Glu-Gly-Ile-Ala-Gly-Gln-D-Arg were obtained from the Protein Research Foundation, Osaka, Japan. Bovine trypsin and chymotrypsin were products of Worthington Biochemical Corp. Chymostatin and elastatinal were gifts from Dr. A. W. Strauss. Polyamide sheets were from Pierce Chemical Co., and all other reagents were of the highest grade commercially available.

Enzyme Assays. The hydrolysis of the *p*-nitroanilide derivatives was followed spectrophotometrically at 385 nm by measuring the rate of liberation of *p*-nitroanilide as previously described (Bundy, 1962, 1963). The hydrolysis of ester substrates was followed spectrophotometrically at 254 nm for BzTyrEE (Hummel, 1959; Kang & Fuchs, 1973) and at 244 nm for TArgME (Hummel, 1959). Substrate concentrations were determined by amino acid analysis after 24- and 72-h acid hydrolyses of aliquots of stock solutions, and enzyme concentrations were determined in the same manner after a 24-h hydrolysis. Substrate stock solutions (1 mg/mL) were prepared with either assay buffer or methanol. Assays were performed in 0.05 M 2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) buffer, pH 8.0, at room temperature on a Gilford recording spectrophotometer. Enzyme assays employing water-insoluble substrates (BzTyrEE and BzTyrNA) contained 10% v/v methanol. It should be pointed out that the presence of organic solvents in the assay mixtures does have some effect on enzyme activity. With increasing solvent concentration the observed enzymatic activity decreases. However, this effect does not appear to differ significantly for any of the enzymes employed. All assays are performed with minimal amounts of solvent and all enzyme assays involving a particular substrate are performed under identical conditions. Human fibroblast collagenase was activated with trypsin as previously described (Stricklin et al., 1977).

Inhibition Assays. The effects of inhibitors on crab protease activity were performed by incubating the enzyme at room temperature with the appropriate inhibitor for the specified time. Stock solutions (10 mg/mL) of TLeuCH_2Cl, TosPheCH_2Cl, and AcAlaPheCH_2Cl were prepared in acetone; ZPheCH_2Cl, chymostatin, and elastatinal were prepared in dimethyl sulfoxide; and TLysCH_2Cl and leupeptin were prepared in assay buffer. Incubations were performed in 0.05 M Tris buffer, pH 8.0. Incubation mixtures employing water-insoluble inhibitors were 10% v/v in either acetone or dimethyl sulfoxide depending on the particular inhibitor used.

Table I: Hydrolysis of Low Molecular Weight Synthetic Substrates

substrate[a]	enzyme	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
BzArgNA	crab protease	0.100	0.021	210
	trypsin	0.093	1.45	15 600
BzValGlyArgNA	crab protease	0.120	3	25 000
	trypsin	0.167	440	2 634 000
BzTyrNA	crab protease	0.263	0.015	57
	chymotrypsin	0.110	0.710	6 500
AcAlaAlaAlaNA	crab protease	0.044	0.002	45
	elastase	0.100	0.091	910
TArgME	crab protease	0.233	0.810	3 500
	trypsin	0.093	46.15	500 000
BzTyrEE	crab protease	1.11	1.09	1 000
	chymotrypsin	0.270	10.20	38 000

[a] Abbreviations used are as presented in the text.

Proteolytic Digests. Enzymatic digests of polypeptide substrates were performed in 0.05 M Tris buffer, pH 8.0, at 37 °C. Enzyme (3% w/w) was added at zero time, and the reaction was stopped after the specified time by acidification to pH 2.0 with 1 N HCl. Enzymatic digests of Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg were performed as previously described (Kobayashi & Nagai, 1978). The rate of hydrolysis of oxidized insulin B chain by crab protease and chymotrypsin was followed by detection of newly formed amino groups with ninhydrin. The amino-terminal cyanogen bromide peptide of ribonuclease A was isolated by chromatography on a column (2.5 × 300 cm) of Sephadex G-75 following cyanogen bromide digestion of the intact protein.

Peptide Separation and Analysis. Peptides from enzymatic digests were separated by ion-exchange chromatography on columns (0.9 × 20 cm) of Dowex 50-X8 as previously described (Bradshaw et al., 1969; Grant & Bradshaw, 1978). Peptides were detected by the ninhydrin reaction following alkaline hydrolysis on an automated peptide analyzer (Hill & Delaney, 1969). Amino acid analyses were performed on a Beckman 119C amino acid analyzer following hydrolysis of peptide or protein samples with 6 N HCl in evacuated sealed tubes at 110 °C for 24 or 72 h. In most cases, the covalent structure of the peptides could be deduced from the amino acid composition. When amino acid composition was not sufficient, amino-terminal residues were identified by the dansyl Edman procedure (Gray, 1972).

Results

Catalytic Activity of Crab Protease with Low Molecular Weight Synthetic Substrates. The amino acid sequence of crab protease (Grant et al., 1980) unequivocally demonstrates that this collagenolytic enzyme is a serine protease related to the mammalian pancreatic serine proteases. It was originally reported (Eisen et al., 1973) that in addition to its collagenolytic activity, crab protease displayed activity toward synthetic low molecular weight esterase substrates commonly used to assay trypsin and chymotrypsin. A comparison of the basic kinetic paramaters (K_m and k_{cat}) of crab protease with those of bovine trypsin, bovine chymotrypsin, and porcine elastase by using several common synthetic esterase and amidase substrates is presented in Table I. The values of K_m and k_{cat} for crab protease with each substrate are compared to those of the corresponding serine protease assayed under identical conditions. These results demonstrate that crab protease is capable of reacting with a variety of substrates, catalyzing the hydrolysis of amide and ester bonds involving both positively charged and aromatic residues. The catalytic efficiency of crab protease, however, is considerably less than that of the other

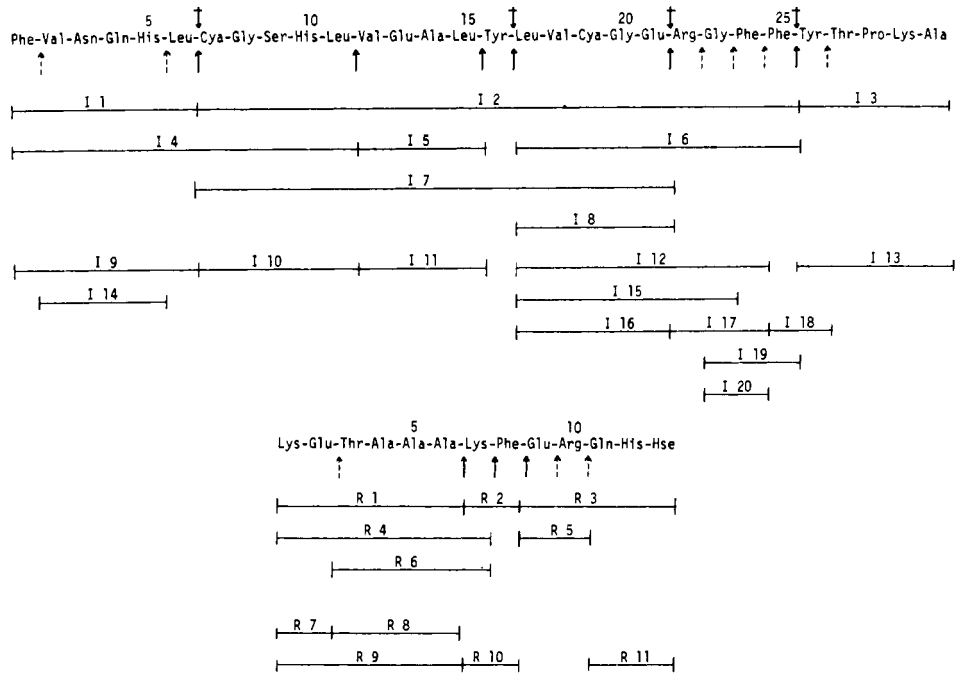

FIGURE 1: Sites of cleavage of bovine oxidized insulin chain B (upper) and the amino-terminal ribonuclease A cyanogen bromide peptide (lower). Solid arrows denote major cleavages and dashed arrows denote minor cleavages. The major cleavages of insulin chain B by chymotrypsin (0.5 h) are shown by crossed arrows. Major cleavages are those which appear early and in relatively high yield. Minor cleavages are those which appeared late or in low yield. Peptides I 1–I 8 and R1–R 6 were isolated from a 0.5-h digestion with crab protease, and peptides I 9–I 20 and R 7–R 11 were isolated from a 20-h digestion with crab protease.

serine proteases with their respective substrates. In addition, the synthetic elastase substrate, AcAlaAlaAlaNA, is also hydrolyzed by crab protease, but with a turnover of ~1 order of magnitude less than that of the trypsin and chymotrypsin substrates. The K_m values of crab protease for the various substrates do not differ significantly from those found with the corresponding serine proteases, the greatest difference being only 4-fold.

Crab protease displays a preference for substrates containing extended peptide chains. The k_{cat} of crab protease with BzArgNA is ~150-fold less than that with BzValGlyArgNA and is consistent with previous observations that secondary binding site interactions contribute significantly to the catalytic efficiency of serine proteases. A similar effect is seen with trypsin employing the same set of substrates.

Peptide Bond Specificity of Crab Protease. The peptide bond specificity of crab protease was investigated by using a variety of noncollagenous polypeptide substrates. The peptides produced from the oxidized B chain of bovine insulin by crab protease after 0.5- and 20-h digestions are presented in Figure 1. The primary sites of cleavage by α-chymotrypsin (0.5 h), determined under identical conditions, are also presented in Figure 1. Based on these results, the primary specificity of crab protease appears to be similar to that of chymotrypsin, prefering bonds where the carbonyl group is donated by phenylalanyl, tyrosyl, and leucyl residues. Four of the six bonds cleaved in high yield by crab protease, including the glutamyl–arginyl bond, are identical with those cleaved by bovine α-chymotrypsin. In addition, crab protease cleaves two additional leucyl bonds in relatively high yield. With extended incubation time, secondary cleavages at glycyl, histidyl, and arginyl residues are also noted. The cleavages at His-5 and Gly-23 appear to be limited to those particular residues since no evidence for cleavage at Gly-8, Gly-20, or His-10 was found. Cleavage at Glu-13 was also not observed. It should also be noted that cleavage at Phe-1, which possesses a free α-amino group, also occurs after extended incubation. No evidence for cleavage between Lys-29 and Ala-30, the carboxyl-terminal residues, was found.

Peptides produced by incubation of crab protease with the 13-residue amino-terminal cyanogen bromide peptide of bovine ribonuclease A are also presented in Figure 1. The major sites of cleavage of this substrate by crab protease appear to be between the Phe–Glu, Lys–Phe, and Ala–Lys bonds. With extended incubation, minor cleavages also appear on the carboxyl-terminal sides of the two glutamyl residues and the single arginyl residue. There is no evidence for cleavage following the amino-terminal lysyl residue. Clevage after Ala-6 is limited to that particular alanyl residue and may be influenced by the adjacent lysyl residue as is often seen for chymotrypsin (Konigsberg & Steinman, 1977). It is also interesting to note that slow cleavage occurs at the two glutamyl residues in this peptide but that cleavage at the Glu–Arg bond appears to be significantly slower than that at an identical bond in bovine insulin oxidized B chain.

Catalytic Rate of Crab Protease against Polypeptide Substrates. The rate of peptide bond cleavage of the polypeptide substrate, oxidized bovine insulin chain B, is presented in Figure 2. These results demonstrate that with extended polypeptide substrates the rate of proteolysis by crab protease closely parallels that of chymotrypsin at initial time points. This is in contrast to the results obtained with low molecular weight synthetic substrates and suggests that extended subsite interaction may be of particular importance for the crab protease. Since the crab protease degrades oxidized insulin chain B more extensively than does chymotrypsin, the distribution of peptide products differs for the two enzymes. For this reason, it is difficult to compare the rates of cleavage at any one bond. For the same reason, based on the appearance of new amino groups, the final degree of cleavage appears greater for the crab protease. However, the relative yields of the major peptides produced by each enzyme are comparable.

*Cleavage of Dnp-Pro-Gln-Gly-Ile-Ala-Gly-Gln-*D*-Arg by Crab Protease.* The synthetic peptide, Dnp-Pro-Gln-Gly-

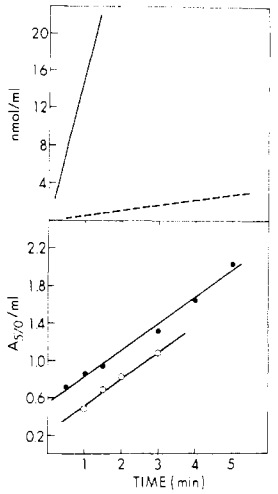

FIGURE 2: Rate of hydrolysis of BzTyrEE and bovine oxidized insulin chain B by crab protease and chymotrypsin. (Top) Hydrolysis of BzTyrEE (1.5×10^{-4} M) by chymotrypsin (—) and crab protease (---). Enzyme concentration was 6.3×10^{-8} M. (Bottom) Hydrolysis of oxidized insulin chain B by chymotrypsin (●) and crab protease (○). Enzyme was 5% w/w of substrate.

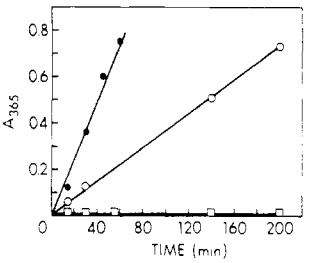

FIGURE 3: Hydrolysis of Dnp-Pro-Glu-Gly-Ile-Ala-Gly-Gln-D-Arg by trypsin-activated human fibroblast collagenase (●), crab protease (○), and chymotrypsin (□). Substrate (1 mg/mL) was digested with 3% w/w enzyme.

Ile-Ala-Gly-Gln-D-Arg, possesses a sequence identical with that in the region of the vertebrate collagenase sensitive Gly-Ile bond in the α_1 chain of type I collagen except that the amino terminal is blocked by a dinitrophenyl group and the carboxyl-terminal residue is the stereoisomer of the naturally occuring L-arginyl residue. This peptide was originally designed as a substrate for enzymes with collagenolytic properties (Masui et al., 1977), and it has been shown that tissue collagenases (Masui et al., 1977; Kobayshi & Nagai, 1978) specifically cleave this peptide at the Gly-Ile bond. It has also been shown that certain gelatinases (Kobayashi & Nagai, 1978; J. L. Seltzer, S. A. Adams, G. A. Grant, and A. Z. Eisen, unpublished results) possess a significant degree of specificity for the Gly-Ile bond in this peptide.

The data presented in Figure 3 demonstrate that this peptide is also a good substrate for crab protease. The rate of hydrolysis of this peptide by crab protease is 30% of the rate found with human skin fibroblast collagenase. In addition, the peptide appears to be totally resistant to digestion by chymotrypsin. Examination of the resulting Dnp-peptide products by amino acid analysis indicates that human skin collagenase cleaves the peptide exclusively at the Gly-Ile bond. Similar examination of the peptide(s) produced by crab protease reveals a different cleavage pattern. In this case, the Dnp-peptide products appear to be an equimolar mixture of Dnp-Pro-Gln and Dnp-Pro-Gln-Gly-Ile. These results indicate that crab protease cleaves this peptide at the Gln-Gly and Ile-Ala bonds rather than at the Gly-Ile bond as seen with vertebrate skin collagenases.

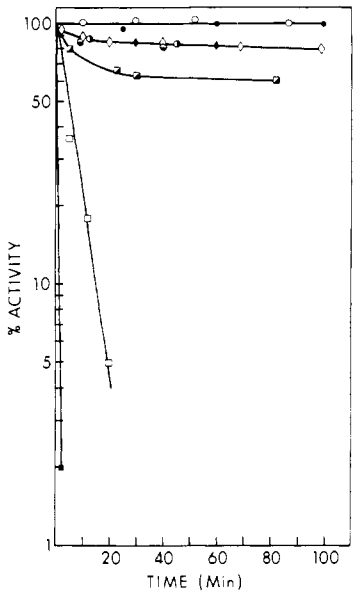

FIGURE 4: Inhibition of crab protease and trypsin. Crab protease (2×10^{-5} M) incubated with a 100-fold molar excess of $TLeuCH_2Cl$ (○), $TosPheCH_2Cl$ (●), $AcAlaPheCH_2Cl$ (♦), $TLysCH_2Cl$ (◇), $ZPheCH_2Cl$ (◪), and leupeptin (◒), a 50-50-fold molar excess of elastatinal (◑), and a 4-fold moar excess of chymostatin (■). Trypsin (2×10^{-5} M) incubated with a 10-fold molar excess of $TLysCH_2Cl$ (□). Crab protease activity was determined on aliquots of the incubation mixtures with BzTyrEE as substrate, and trypsin activity was determined with BzArgNA as substrate.

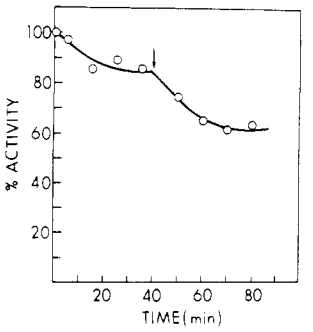

FIGURE 5: Time course of inhibition of crab protease by $TLysCH_2Cl$. Crab protease (2×10^{-5} M) was incubated with a 100-fold molar excess of $TLysCH_2Cl$. At the time designated by the arrow, an equal aliquot of $TLysCH_2Cl$ was added. Enzyme activity was determined with BzTyrEE as substrate.

Effect of Specific Protease Inhibitors on Crab Protease. The specificity properties of crab protease were also studied by investigating the effects of various site-specific protease inhibitors. It was originally shown that substances such as ethylenediaminetetraacetic acid and cysteine, inhibitors of the metalloenzyme collagenases, had little or no effect on the collagenolytic activity of the crab enzyme but that some inhibitors of serine proteases were effective (Eisen et al., 1973). These include diisopropyl fluorophosphate, soybean trypsin inhibitor, and $TLysCH_2Cl$. $TosPheCH_2Cl$ had no effect on crab protease.

This observation has been confirmed and expanded to include a wider range of inhibitors, particularly in regard to specific inhibitors of chymotrypsin (Figure 4). $TosPheCH_2Cl$ and $TLeuCH_2Cl$ are without effect even at a 100-fold molar excess of inhibitor to enzyme. Substitution of the tosyl group for less bulky substituents does produce some inhibition as demonstrated with $ZPheCH_2Cl$ and $AcAlaPheCH_2Cl$, although it is less than optimal. $TLysCH_2Cl$ also appears to be marginally inhibitory under the conditions used.

The inhibition of crab protease by the chloromethyl ketone inhibitors tested is not first order as would be expected for a site-specific process such as the inhibition of trypsin with $TLysCH_2Cl$ (Figure 4) but rather reaches a plateau where no further inhibition is observed. The addition of a second aliquot of inhibitor at this point (Figure 5) produces a similar pattern where an initial increase in inhibition is followed by a plateau at substantially less than complete inhibition. The degree of inhibition at the plateau is dependent on the initial amount of inhibitor present. The partial inhibition observed with these substances is not due to spontaneous hydrolysis of the inhibitor since preincubation of the inhibitor, prior to the addition of enzyme, produces identical results. Furthermore, this effect does not appear to be the result of catalytic degradation of the inhibitor by the protease. Preincubation of $TLysCH_2Cl$ with crab protease does not reduce the effective concentration of inhibitor as judged by its ability to subsequently inhibit trypsin. If the inhibitor was being degraded by crab protease to an extent that would produce the inhibition plateau effect in Figures 4 and 5, the inactivation of added trypsin should proceed at a significantly reduced rate and the maximum inhibition possible in the presence of excess trypsin should be significantly decreased. $TLysCH_2Cl$ preincubated with crab protease inhibits added trypsin at a rate identical with that of an equal concentration of untreated $TLysCH_2Cl$. When the inhibitor is present in excess of trypsin, the inhibition occurs as a first-order process. When trypsin is present in excess of inhibitor, the rate of inhibition gradually decreases to 0 as the inhibitor is consumed. The final level of inhibition corresponds to the mole ratio of inhibitor to enzyme and is independent of the presence of the crab protease.

The apparent inhibition of crab protease by $TLysCH_2Cl$ is also reversible to a large extent in the presence of substrate. This effect is most apparent when crab protease is assayed with a relatively poor substrate such as BzTyrEE and can be seen as an accelerating rate of hydrolysis of substrate. It thus appears that the small degree of inhibition of crab protease by $TLysCH_2Cl$ is due to the reversible binding of inhibitor at or near the active site without subsequent alkylation of an active-site residue. When BzTyrEE is used as substrate, the final rate of hydrolysis is less than that for untreated enzyme and thus results in an apparent low level of inhibition. When a more efficient substrate such as BzValGlyArgNA is used, it is difficult to observe any appreciable inhibition of crab protease by $TLysCH_2Cl$. This observation probably reflects a much faster displacement of reversibly bound inhibitor than in the case with BzTyrEE. Similar effects are seen with $ZPheCH_2Cl$ and $AcAlaPheCH_2Cl$.

Leupeptin, elastatinal, and chymostatin, protease inhibitors of microbial origin (Umezawa, 1976), were also tested for their ability to inhibit crab protease. The results of these studies are also presented in Figure 4. Of these substances, only chymostatin displayed significant inhibition at relatively low ratios of inhibitor to enzyme concentration. Chymostatin, an effective inhibitor of chymotrypsin, contains a carboxyl-terminal L-phenylalaninal residue essential to its specificity and inhibitory capacity. The corresponding inhibitors of trypsin and elastase, leupeptin and elastatinal, which contain carboxyl-terminal L-argininal and L-alaninal residues, respectively, displayed only a small degree of inhibition of crab protease even at much higher concentrations. The inhibition of crab protease by leupeptin and elastatinal gradually reaches a plateau similar to that observed with the chloromethyl ketones (Figure 4). Inhibition by chymostatin is at its maximum value at the earliest measureable time point and is linearly dependent on chymostatin concentration in a stoichiometric manner.

Discussion

The native helical structure of collagen is extremely resistant to proteolysis by most proteases present in mammalian tissues. Collagenases, by definition, are those enzymes capable of cleaving the helical portion of collagen under physiological conditions of pH, temperature, and ionic strength. Collagenases in general are metal-requiring endopeptidases which cleave collagen across its three polypeptide chains at a locus approximately three-fourths of the distance from the amino terminus of the molecule. Studies employing vertebrate collagenases have shown that the cleavage sites in type I collagen are at a specific Gly-Ile bond in the α_1 chain and a corresponding Gly-Leu bond in the α_2 chain (Gross et al., 1974; Highberger et al., 1979). It is not known, however, if the specificity for this cleavage resides exclusively in the collagenase or whether the collagen molecule itself also has inherently greater susceptibility to cleavage at this point. The observation that many other proteases of rather broad proteolytic capabilities are ineffective in cleaving collagen and the fact that most metal-dependent collagenses are specific for collagen as opposed to other protein substrates support the idea that collagenase selectivity is due to the enzyme's ability to interact with the substrate in a very precise manner.

Crab protease, a serine protease related to the pancreatic serine proteases, possesses the unique ability, among this class of enzymes, to cleave the native triple helix of collagen in a location similar to the vertebrate tissue collagenases. Although their physiological roles appear to be quite different, the observation that a serine protease and a metalloenzyme have in common the ability to cleave helical collagen suggests that they may also possess certain common structural features particularly in the regions of their substrate binding sites.

In contrast to the vertebrate collagenases, crab protease is a good general protease capable of extensively degrading a wide variety of protein substrates as well as a number of low molecular weight synthetic esterase and amidase substrates commonly used to assay for trypsin, chymotrypsin, and elastase. The peptide bond specificity of crab protease appears to be rather broad and cleaves on the carboxyl-terminal side of an unusual variety of amino acid residues including those with both positively and negatively charged side chains as well as hydrophobic side chains. Crab protease appears to favor tyrosyl, phenylalanyl, and leucyl residues, similar to chymotrypsin, and is capable of cleaving polypeptide substrates at these residues at a rate comparable to chymotrypsin. However, at least one example each of highly efficient cleavages at lysyl and alanyl residues was observed as well as repeated examples of slower cleavages at arginyl and glutamyl bonds. In addition, crab protease cleaved the Dnp-peptide following a glutaminyl and isoleucyl bond. Cleavage at the glutaminyl bond is perhaps consistent with a chymotrypsin-like specificity, but the isoleucyl cleavage seems unusual as do the low levels of cleavage observed at certain histidyl and glycyl residues.

The rather broad range of specificity observed for crab protease when polypeptides are employed as substrates is also observed with low molecular weight esterase and amidase substrates. In all cases studied, however, the rate of hydrolyis is considerably less than the more selective serine proteases, trypsin and chymotrypsin. This is in contrast to the observation that with elongated polypeptide substrates the rate of hydrolysis catalyzed by crab protease is comparable to that of chymotrypsin. The observation that the rate of hydrolysis of synthetic substrates by most serine proteases increases with increasing peptide length, due to secondary binding interactions

	212	216	218A	220
Collagenase	-Ile-Thr-Ser-Phe-	Gly-	Ala-Ala-Ala-	Gly-Cys-
Chymotrypsin B	-Ile-Val-Ser-Trp-	Gly-	Ser-Ser-	-Thr-Cys-
Trypsin	-Ile-Val-Ser-Trp-	Gly-	Ser-	-Gly-Cys-
Elastase	-Val-Thr-Ser-Phe-	Val-	Ser-Arg-Leu-	Gly-Cys-

FIGURE 6: Comparison of the substrate binding region of serine proteases in the area of Ser-Trp-Gly-216 in chymotrypsin. The numbering system is that for chymotrypsin.

(Poulos et al., 1976), is also seen with crab protease (Table I).

The interpretation of the exact nature of these interactions and the factors determining substrate specificity and catalytic rate at a structural level rely to a large extent on knowledge of the tertiary structure of crab protease. However, the models developed for substrate binding with other serine proteases provide some basis for interpretation of these results. The models for polypeptide substrate binding for chymotrypsin (Segal et al., 1971) and subtilisin (Robertus et al., 1972) are similar. Both enzymes bind the polypeptide substrate in the same manner through an extended segment of backbone chain in the enzyme, Ser-Trp-Gly-216 in chymotrypsin, and Ser-Leu-Gly-127 in subtilisin, by forming an antiparallel β-pleated sheet with the substrate. The plane of this backbone chain comprises one wall of the crevice that binds the aromatic side chain at P_1[1] in both enzymes. If S_3 were other than glycine, its side chain would block the S_1 crevice in both enzymes, as is the case for elastase (Shotton & Watson, 1970). Crab protease contains a similar segment, Ser-Phe-Gly-216, in the corresponding location (Grant et al., 1980). However, in contrast to trypsin and chymotrypsin, crab protease contains the small side-chain residues Ala-Ala-Ala-218A (chymotrypsin numbering, see Figure 6) at what could be additional subsites. In elastase these residues are present, Ser-Arg-Leu-218A, but are more bulky and, compared to chymotrypsin and trypsin, comprise a one- and two-residue insertion at this point. The cleavages of elastin by elastase and collagen by collagenase both appear to be dependent to some extent on the recognition of conformational features in their substrates rather than on any unique features of sequence. Both crab protease and elastase contain this extended segment of polypeptide chain which may be functioning similarly, although perhaps uniquely for each substrate, in the recognition and binding process. Thus, the Ala-Ala-Ala sequence in crab protease may represent an extended region for substrate interaction over what occurs in in trypsin and chymotrypsin. If this is the case, it may explain the observation that crab protease appears to favor long segments of polypeptide and may be crucial for its unique interaction with collagen. Accordingly, a region of extended substrate interaction with the enzyme may also lead to more efficient cleavage of certain bonds, due to better binding, that normally would not be as susceptible strictly on the basis of selectivity at P_1. This might broaden the primary specificity of an enzyme and is consistent with what is observed with crab protease.

The observation that crab protease effectively utilizes small molecular weight synthetic substrates containing both the positively charged arginyl residue and the bulky hydrophobic tyrosinyl residue suggests that the binding site of crab protease may be less constrained than that for either trypsin or chymotrypsin and thus less selective. The observation that hydrolysis of these substrates is at a much slower rate than that for tyrpsin and chymotrypsin may be due primarily to a lack of extended secondary interaction of crab protease with these substances.

The fact that none of the chloromethyl ketones employed, although they contain amino acid residues that were clearly selected by crab protease in polypeptide and synthetic substrates, appear to be capable of alkylating an active-site nucleophile suggests that a nucleophile was not in close enough proximity to the methylene carbon. Although the charge-relay system apparently must be maintained for catalytic activity, the binding geometry may be such so as not to allow alkylation by these compounds. This is consistent with the fact that chymostatin is a very effective inhibitor of crab protease, since the productive step for inhibition with chymostatin is formation of a hemiacetal with the active-site serine and thus does not rely on subsequent alkylation of the active-site histidyl residue. This is also consistent with the suggested inactivation mechanism of chloromethyl ketones (Poulos et al., 1976) in that the rate-limiting step is alkylation and that in the absence of alkylation, the formation of the hemiketal with the active-site serine is reversible. The observation that chymostatin is a much more effective reversible inhibitor than $TLysCH_2Cl$, for example, may be due simply to differences in binding characteristics due to the extended peptide-like structure of chymostatin. The ineffectiveness of leupeptin and elastatinal also suggests a marked preference for a phenylalanine side chain.

Alternatively, the lack of alkylation of crab protease by the chloromethyl ketones may be similar to, but not identical with, what has been observed with subtilisin (Robertus et al., 1972). $ZPheCH_2Br$ is a good subtilisin inhibitor while $TosPheCH_2Cl$ is totally ineffective. On the other hand, subtilisin does hydrolyze tosyl-L-phenylalanine ethyl ester but more slowly than benzyloxycarbonyl-L-phenylalanine ethyl ester. Model building demonstrated that, if the methylene carbon linkage to His-64 was maintained, a stereochemically permissible tosyl group of P_2 could not be constructed. This was not the case for the more flexible and less bulky benzyloxycarbonyl group. When the methylene linkage is not present, as for hydrolysis of tosyl-L-phenylalanine ethyl ester, a sterically acceptable acyl intermediate could be constructed.

Both $TosPheCH_2Cl$ and $TLeuCH_2Cl$ are totally ineffective against crab protease, but TArgME does serve as a substrate. Replacing the tosyl group of $TosPheCH_2Cl$ with the benzyloxycarbonyl group does allow some interaction with the enzyme, although it does not appear to be productive for alkylation. $TLysCH_2Cl$ also appears to interact in some way with crab collagenase. However, the exact nature of its interaction with the enzyme is not known, and no evidence for significant alkylation is found.

Although the bond specificity of crab protease is different than that of the vertebrate metalloenzyme collagenases, crab protease does catalyze the cleavage of the synthetic collagenase peptide on either side of the Gly-Ile bond, while the peptide is not cleaved by chymotrypsin. It must be pointed out, however, that this does not constitute proof of collagenolytic activity since the short peptide is not in helical conformation. The differences in the cleavage patterns observed with these two collagenases are consistent with the fact that the human skin collagenase is a neutral protease while crab protease is a serine protease related to the mammalian pancreatic serine proteases. It is not possible to tell on the basis of this observation if this cleavage pattern also holds for helical collagen, but these results, along with the observation that crab protease cleaves helical collagen in a single specific and limited area of the molecule, are consistent with the suggestion (Gross & Nagai, 1965; Weiss, 1976; Highberger et al., 1979) that the

[1] For definition of the notation P_1, P_2, ... and S_1, S_2, ..., see Schecter & Berger (1967).

structure of collagen in the area of the cleavage site may be of a less tightly coiled nature and perhaps possess a unique conformation which acts synergistically with these particular enzymes in producing the observed proteolysis. In this regard, the conformational nature of the active site of crab protease and the regions involved in substrate binding are of particular interest. Furthermore, investigations into the nature of the interaction of crab protease with collagen may lead to a better understanding of the structural features of collagen that are responsible for its degradation. Crystallographic and chemical modification studies designed to elucidate these points are now in progress.

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