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A Collagenolytic Serine Protease with Trypsin-like Specificity from the Fiddler Crab *Uca pugilator*†

Gregory A. Grant,* James C. Sacchettini, and Howard G. Welgus

ABSTRACT: A second collagenolytic serine protease has been isolated from the hepatopancreas of the fiddler crab, *Uca pugilator*. This enzyme cleaves the native triple helix of collagen under physiological conditions of pH, temperature, and ionic strength. In addition to its collagenolytic activity, the enzyme exhibits endopeptidase activity toward other polypeptides and small molecular weight synthetic substrates. The polypeptide bond specificity of this enzyme is similar to that of bovine trypsin as is its interaction with specific protease inhibitors. The amino-terminal sequence of this enzyme displays significant homology with other serine proteases, most notably with that of crayfish trypsin, and demonstrates that this enzyme is a member of the trypsin family of serine endopeptidases. The relatively unique action of this protease with regard to both collagenous and noncollagenous substrates has important implications concerning the specificity and mechanism of collagen degradation.

The trypsin-related serine proteases comprise one of the best characterized families of proteins which have evolved from a common ancestor. Serine proteases from many sources display a marked degree of similarity in primary structure (Woodbury et al., 1978) and share common elements of three-dimensional structure and mechanism of action (Blow et al., 1969; Shotton & Watson, 1970; Stroud et al., 1971). Despite these similarities, it is clear that serine proteases function in a variety of diverse and often highly specific physiological roles. The most well studied of these are the enzymes from vertebrate systems which participate in such processes as digestion, blood coagulation, fertilization, and the immune response. Although serine proteases from invertebrate sources have been known for some time, they are less well studied, and their specific functions have been less well-defined. Recently, a new group of invertebrate serine proteases, which possess the ability to cleave the native triple helix of collagen under physiological conditions of pH, temperature, and ionic strength, has become recognized. These enzymes are the first serine proteases known to possess collagenolytic activity and as such represent a new group of collagenases. However, unlike the metalloenzyme collagenases of mammals, these invertebrate collagenases are involved in digestion rather than morphogenesis.

Collagenolytic serine proteases have been reported 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 to be reported and the most extensively characterized of these is crab protease I from the fiddler crab. Its complete amino acid sequence has been determined and shown to possess significant homology to the trypsin-related serine proteases of mammals (Grant et al., 1980). Furthermore, its specificity with both collagenous and noncollagenous substrates has been documented (Grant & Eisen, 1980; Grant et al., 1982; Welgus et al., 1982). Crab protease I successfully degrades all five types of native collagen and is a good general protease of noncollagenous polypeptides. The enzyme appears to be a chymotrypsin-like enzyme in that it displays a significant degree of preference for phenylalanyl, tryptophanyl, and leucyl residues although it has also been shown to cleave at both positively and negatively charged residues as well. It is specifically inhibited by chymostatin while leupeptin is without effect.

In addition to crab protease I, the hepatopancreas of the fiddler crab contains at least one additional distinct collagenolytic serine protease. The present study details the purification and characterization of crab protease II and demonstrates that its specificity, at least with respect to noncollagenous substrates, is markedly different from that of crab protease I and is very similar to that of bovine trypsin.

Materials and Methods

N-Benzoyl-L-valylglycyl-L-arginine *p*-nitroanilide (BzValGlyArgNA), *N*-benzoyl-L-tyrosine ethyl ester (BzTyrEE), N^{α}-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), *N*-benzyloxycarbonyl-L-phenylalanine chloromethyl ketone (ZPCK), and bovine insulin were from Sigma Chemical Co. *N*-benzoyl-L-arginine *p*-nitroanilide (BzArgNA) was from Vega Biochemicals. Bovine trypsin was a product of Worthington Biochemical Corp., and all sequencing reagents were from Beckman. All other reagents were of the highest grade commercially available.

Bovine insulin oxidized chain B was produced from bovine insulin by performic acid oxidation (Weber et al., 1972) and separated from the oxidized A chain by high-pressure liquid chromatography on a Varian Model 5020 liquid chromatograph. A Beckman ultrasphere octadecyl reverse-phase column (4.6 × 250 mm) was used with a linear gradient of 1-propanol (1%/min) in 15 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.1 at 30 °C. The proteolytic products of crab protease II digestion of oxidized insulin chain B were separated in the same manner.

Proteolytic digests of polypeptide substrates were performed in 0.05 M Tris buffer, pH 8.0, at room temperature. Enzyme

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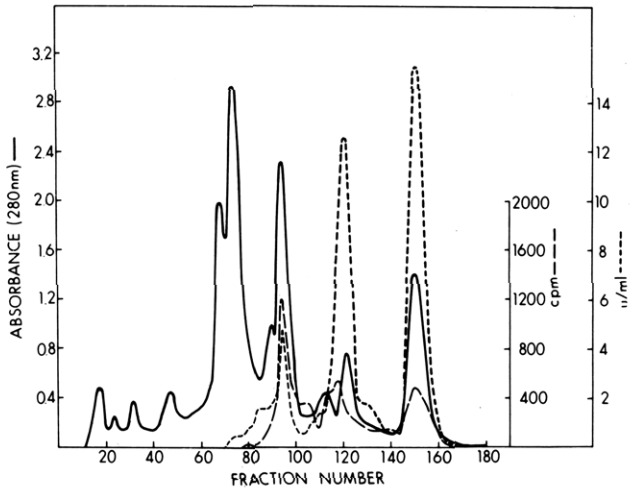

FIGURE 1: DEAE-Sepharose CL6B chromatography. The active pool from AcA-34 gel filtration was chromatographed on a column (2.5 × 28 cm) of DEAE-Sepharose CL6B equilibrated with 0.05 M Tris buffer, pH 7.5. The column was developed with a 2-L linear gradient from 0 to 1 M NaCl at a flow rate of 45 mL/h. Fractions of 12 mL were collected and monitored for absorbance at 280 nm (—), collagenolytic activity (--), and amidase activity (---) with BzValGlyArgNA.

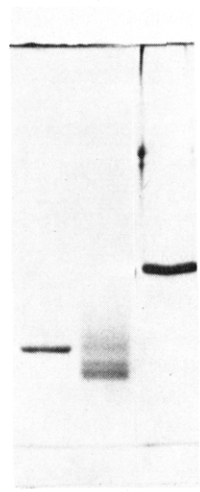

FIGURE 2: Gel electrophoresis of crab proteases. The G-100 pools of crab proteases I and II were electrophorsed on 12% polyacrylamide gels in the presence of $NaDodSO_4$. Left lane, crab protease I; middle lane, crab protease II; right lane, crab protease II in the presence of dithiothreitol.

(2% w/w) was added at zero time, and aliquots were taken for analysis at the specified time.

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the method of King & Laemmli (1971) on slab gels. Following electrophoresis, the protein bands were stained with 1% Coomassie blue.

Amino acid analyses were performed on a Beckman 119C amino acid analyzer following hydrolysis with 6 N HCl in evacuated sealed tubes at 110 °C for 24 h. Enzyme assays and inhibition assays were performed as previously described (Grant & Eisen, 1980). Collagenolytic activity was measured by the release of [^{14}C]glycine-containing peptides from native reconstituted guinea pig skin collagen fibrils as previously described (Nagai et al., 1966; Grant et al., 1982).

Amino-terminal sequence analysis was performed by automated Edman degradation on a Beckman Model 890C sequencer using 1 M Quadrol buffer. Sequencer fractions were converted from the phenylthiazolinones to the phenylthiohydantoins by treatment with 1 N HCl for 10 min at 80 °C and analyzed by thin-layer chromatography (Jacobs & Niall, 1975) and high-pressure liquid chromatography. Analysis of PTH-amino acids by high-pressure liquid chromatography was performed with a Beckman ultrasphere octadecyl-PTH reverse-phase column by a modification of the method of Johnson et al. (1979). The column is equilibrated with 16.7 mM sodium acetate, pH 5.5, and the PTH-amino acids are eluted with a methanol/acetonitrile (17:3) mixture according to the following program: 0–5 min, 34%; 5–6 min, 34–50%; 6–16 min, 50%; 15–18 min, 50–34%; and 18–20 min, 34%. The column is run at 34 °C and a constant flow rate of 1.5 mL/min. All amino acids, including *S*-(carboxymethyl)cysteine, can be resolved and the next sample injected in less than 20 min.

Results

Crab Protease II Isolation. Crab protease II was purified from an acetone powder of fiddler crab hepatopancreata in a manner similar to that described for crab protease I (Eisen et al., 1973; Grant et al., 1982). The enzymatic activity is extracted from the acetone powder of the hepatopancreata and chromatographed on Ultra-gel ACA-34 (LKB) in the usual manner. The single activity peak from this column is then chromatographed on DEAE-Sepharose CL6B (Pharmacia), which separates the individual activities into distinct fractions. As can be seen in Figure 1, essentially three peaks of activity are resolved by this procedure. The first peak of activity (fractions 90–102) contains crab protease I (Grant et al., 1982; Grant & Eisen, 1980). The second activity peak (fractions 110–130) contains both collagenolytic and tryptic activity; however, the two activities are not coincident, and they have not yet been further purified. Crab protease II is found in the third peak (fraction 144–160). It elutes as a symmetrical peak where the absorbance at 280 nm, the collagenolytic activity, and the tryptic activity are coincident. The protein is usually pure at this point, but it can be chromatographed on hydroxylapatite and Sephadex G-100 as originally described for crab protease I to remove any impurities that may be present. The protein elutes from the G-100 column as a single symmetrical peak.

Polyacrylamide disc gel electrophoresis of the Sephadex G-100 product in the presence of sodium dodecyl sulfate ($NaDodSO_4$) shows a diffuse pattern of protein staining which migrates with an apparent molecular weight of 23 000–26 000 (Figure 2). Electrophoresis of the same material in the presence of β-mercaptoethanol or dithiothreitol results in a single sharp protein band. However, this band appears at a position on the gel corresponding to a molecular weight of approximately 40 000. This atypical behavior on $NaDodSO_4$ gels and the effect of sulfhydryl reagents are not understood. It may be due to reagent-induced dimerization although the treated protein appears somewhat smaller than that which would be expected for the dimer. There is some evidence of trace amounts of larger molecular weight aggregates at low reagent concentrations, but the major band remains at 40 000 over a broad range of sulfhydryl concentrations. The extremely acidic nature of this protein ($pI < 3$) may contribute in some way to this observation, but whatever the cause of this anomalous behavior, the single, sharp protein band resulting from exposure to sulfhydryl reagents would suggest that a single molecular weight species is present. This is supported by the fact that Edman degradation of this product yields a single sequence (vide infra). Furthermore, gel filtration chromatography data, where crab protease II elutes coincident with crab protease I on both ACA-34 and Sephadex G-100, support

Table I: Amino Acid Composition of Crab Protease II[a]

amino acid	24 h	48 h	72 h	calcd value	integral value	crab protease I (Grant et al., 1980)
aspartic acid	31.3	32.2	32.0	31.8	32	28
threonine	10.8	11.9	12.5	11.7	12	22
serine	22.5	21.9	21.6	22.9	23[b]	14
glutamic acid	28.5	29.4	29.3	29.1	29	12
proline	8.5	8.8	9.3	8.9	9	12
glycine	40.0	37.9	37.5	38.5	39	27
alanine	20.4	19.3	19.4	19.7	20	20
half-cystine	12.0[c]				12	6
valine	9.2	6.9	6.0	7.4	7	24
methionine	2.0[d]				2	3
isoleucine	7.6	8.1	8.1	7.9	8	17
leucine	14.0	15.9	15.9	15.3	15	13
tyrosine	10.8	10.7	10.6	10.7	11	7
phenylalanine	8.4	8.4	8.5	8.4	8	8
histidine	4.3	3.8	4.0	4.0	4	4
lysine	2.1	1.8	2.0	2.0	2	1
arginine	2.7	2.1	2.1	2.3	2	4
tryptophan					7[e]	4
total					242	226

[a] Residues per molecule, M_r 24 000. [b] Extrapolated to zero time of hydrolysis. [c] Determined as cysteic acid after performic acid oxidation. [d] Determined as methionine sulfone after performic acid oxidation. [e] Determined spectrophotometrically (Edelhoch, 1967).

Table II: 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 II	0.0069	2.0	290 000
	bovine trypsin[c]	0.093	1.45	15 600
	crab protease I[c]	0.100	0.02	210
BzValGlyArgNA	crab protease II	0.0016	26.8	16 750 000
	bovine trypsin[c]	0.167	440	2 634 000
	crab protease I[c]	0.120	3.0	25 000
BzTyrEE	crab protease II	ND[b]	ND	ND
	bovine chymotrypsin[c]	0.27	10.20	38 000
	crab protease I[c]	1.11	1.09	1 000

[a] Abbreviations are as presented in the text. [b] No detectable activity. [c] Grant & Eisen (1980).

the view that the true molecular weight of crab protease II is very similar to that of crab protease I.

Amino Acid Analysis. The amino acid composition of crab protease II is given in Table I. The results are expressed as residues per mole and based on a molecular weight of 24 000. For comparison, the amino acid composition of crab protease I is also presented, and several significant differences are apparent. These are the increased levels of serine, glutamic acid, glycine, and half-cystine and the decreased levels of threonine, valine, and isoleucine in crab protease II. Perhaps the most notable feature of these compositions is the similarly low level of basic amino acids in both proteins. This is consistent with the highly acidic nature of each as reflected in their elution behavior on DEAE-Sepharose. Furthermore, the increased levels of acidic residues in crab protease II, particularly glutamic acid, are consistent with its later elution from DEAE-Sepharose as compared to crab protease I. A second notable feature is the presence of 12 half-cystine residues in crab protease II as opposed to 6 half-cystines in crab protease I. This would suggest that crab protease II contains six disulfide bonds, which is the same number found in bovine trypsin.

Collagenolytic Activity of Crab Protease II. The action of crab protease II on native chick skin type I collagen in solution is shown in Figure 3. The major early-cleavage products migrate in the area of the three-quarter-length TC^A products produced by mammalian collagenase. However, unlike mammalian collagenases, crab protease II produces multiple products in the TC^A area, suggesting cleavage at more than one site on each α chain. In addition to degrading collagen in solution, crab protease II also degrades fibrillar collagen at 37 °C.

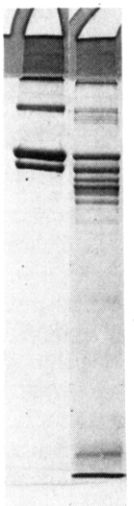

FIGURE 3: Collagen degradation by crab protease II. Chick skin type I collagen (25 μg) was incubated with crab protease II (9 μg) at 25 °C for 1 h. The reaction was stopped with leupeptin. Left lane, collagen without added enzyme; right lane, collagen plus crab protease II.

Catalytic Activity of Crab Protease II with Low Molecular Weight Substrates. A comparison of the basic kinetic parameters (K_m and k_{cat}) of crab protease II with those of crab

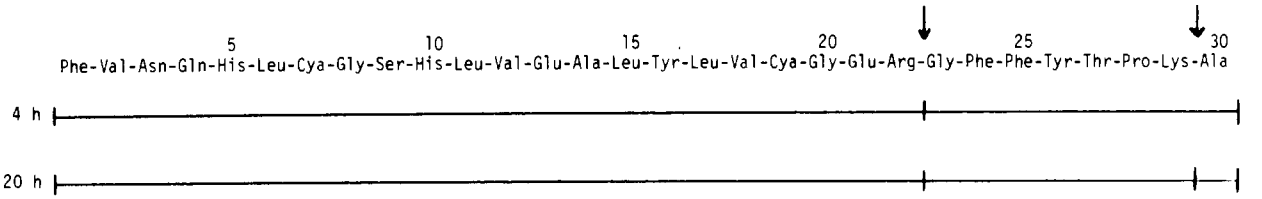

FIGURE 4: Peptide bond specificity of crab protease II. Sites of cleavage of bovine oxidized insulin chain B by crab protease II are indicated by arrows.

protease I, bovine trypsin, and bovine chymotrypsin is presented in Table II. The K_m of crab protease II for both *N*-benzoyl-L-arginine *p*-nitroanilide and *N*-benzoyl-L-valylglycyl-L-arginine *p*-nitroanilide is approximately 1–2 orders of magnitude less than that seen for either bovine trypsin or crab protease I. The turnover rate of crab protease II is about the same as trypsin with benzoyl-L-arginine *p*-nitroanilide and approximately 16-fold less than trypsin for benzoyl-L-valylglycyl-L-arginine *p*-nitroanilide. Crab protease I exhibits a significantly lower turnover than either crab protease II or trypsin with both substrates. However, all three enzymes exhibit an increased selectivity (k_{cat}/K_m) for benzoyl-L-valylglycyl-L-arginine *p*-nitroanilide. Crab protease II, on the other hand, is inactive toward the chymotrypsin substrate *N*-benzoyl-L-tyrosine ethyl ester, which is a substrate for crab protease I. Furthermore, crab protease II is effectively inhibited by leupeptin and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone but not by chymostatin or *N*-benzyloxycarbonyl-L-phenylalanine chloromethyl ketone. This pattern is similar to that of bovine trypsin.

Peptide Bond Specificity of Crab Protease II. The specificity of crab protease II against polypeptide substrates was tested with the oxidized B chain of bovine insulin. As shown in Figure 4, crab protease II cleaves exclusively on the carboxyl side of the basic amino acids arginine and lysine. After 4 h of incubation, the starting peptide is approximately 70% degraded, and only two peptides, resulting from cleavage at Arg-22, are evident. After 20 h of incubation, the only additional cleavage that has occurred is at Lys-29, resulting in the complete conversion of Gly-23–Ala-30 to Gly-23-Lys-29. No evidence of even limited cleavage at any other residues is detectable. The slower cleavage at Lys-29 as opposed to Arg-22 is probably due to the fact that Lys-29 is penultimate rather than being due to an increased selectivity for arginine. Reduced rates of cleavage at terminal polypeptide bonds are a common observation with endopeptidases.

Amino-Terminal Sequence of Crab Protease II. The amino-terminal sequence of the first 20 residues of crab protease II is shown in Figure 5 along with the amino-terminal sequences of several other serine proteases. It is clear from these data that crab protease II is related to this group of proteases. Although a significant degree of homology is apparent with a wide variety of serine proteases, the most striking comparison is that of crab protease II to crayfish trypsin, which display 80% identity to each other.

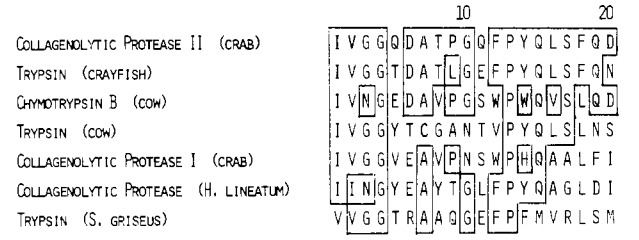

FIGURE 5: Amino-terminal sequence of crab protease II. Comparison of amino-terminal sequences of crab protease II with those of several other serine proteases. Bovine trypsin and chymotrypsin B are from Woodbury et al. (1978), crayfish trypsin is from Zwilling et al. (1975), crab protease I is from Grant et al. (1980), *S. griseus* trypsin is from Olafson et al. (1975), and the collagenolytic protease from *Hypoderma lineatum* is from Lecroisey et al. (1979).

Discussion

This report describes the isolation and some of the properties, particularly against noncollagenous substrates, of a second serine protease from the hepatopancreas of the fiddler crab which possesses the relatively unique ability to cleave peptide bonds in the native triple helix of collagen. This enzyme is one of a growing number of invertebrate serine proteases which have in the last few years been recognized as collagenolytic. As such, these enzymes constitute a new functional group of serine proteases and a new type of collagenase. That is, they are members of the trypsin family of endopeptidases which function to degrade collagen in a digestive rather than a morphogenetic role.

Unlike crab protease I, which migrates as a single band on $NaDodSO_4$ gel electrophoresis, crab protease II exhibits anomalous behavior during electrophoresis. The underlying reasons for this behavior are not understood, but the remainder of the experimental evidence supports the contention that the protein as isolated is a homogeneous species. This evidence includes the single amino-terminal sequence, the similarity in amino acid composition to crab protease I especially among the basic residues, the elution of crab protease II as a single symmetrical peak on ion-exchange and gel filtration chromatography, the single band on $NaDodSO_4$ gels in the presence of sulfhydryl reagents, and the presence of only a single highly specific proteolytic activity. Furthermore, the molecular weight of crab protease II is indistinguishable from that of crab protease I as determined by gel filtration.

Crab protease II, in addition to its collagenolytic activity, is remarkably similar to bovine trypsin in its peptide bond specificity and its response to specific protease inhibitors. Crab protease II is highly specific in its preference for arginyl and lysyl residues at the P_1 position in low molecular weight synthetic substrates and inhibitors as well as in polypeptide substrates. While the turnover of crab protease II with BzValGlyArgNA is approximately 16-fold lower than that of bovine trypsin, the K_m of crab protease II with this substrate is approximately 100-fold less. These two factors taken together, reflected in the ratio of k_{cat} to K_m, demonstrate that crab protease II is a very efficient trypsin-like enzyme. Furthermore, its interaction with the naturally occurring microbial protease inhibitors leupeptin and chymostatin confirms the trypsin-like specificity of this protease. Leupeptin and chymostatin are highly effective low molecular weight polypeptide inhibitors of trypsin and chymotrypsin, respectively. Leupeptin contains a carboxyl-terminal L-argininal residue and chymostatin contains a carboxyl-terminal L-phenylalaninal residue which are essential to their specificity and inhibitory capacity. Like bovine trypsin, crab protease II is rapidly inhibited by leupeptin while chymotrypsin is without effect. This is in contrast to chymotrypsin and crab

protease I which are inhibited only by chymostatin.

Comparison of the amino-terminal sequence of crab protease II with that of other serine proteases presents a somewhat different picture, however. Although crab protease II displays 80% identity to crayfish trypsin over the segments analyzed, its similarity in amino-terminal sequence to chymotrypsin and several of the blood clotting factors is more striking than its similarity to the other trypsins and collagenolytic proteases for which this information is available. However, these comparisons are made with segments representing only about 10% of each polypeptide and, therefore, could vary considerably from comparison of the complete sequences.

The similarity between crab protease II and crayfish trypsin is further supported by the fact that both are strongly acidic crustacean serine proteases with very similar peptide bond specificity. Crayfish trypsin has not been investigated with regard to collagenolytic activity, but the remarkable similarity between these two enzymes would suggest that crayfish trypsin may well possess such activity.

The crab collagenolytic proteases represent a highly efficient system for the digestion of all manner of ingested protein. Perhaps more importantly, however, the action of these enzymes suggests certain considerations concerning the mechanism of collagenolysis in general. In all enzyme-mediated proteolytic events, the initial event is the formation of an enzyme–substrate complex which leads to the proper orientation at the active site for peptide bond cleavage. The formation of this productive E–S complex may depend to some extent on the enzyme's specificity for a particular amino acid residue inasmuch as that residue may contribute to the binding process. However, cleavage will not occur at any particular residue if proper orientation is not first achieved. Crab protease II and bovine trypsin display a similar peptide bond specificity, yet only crab protease II cleaves helical type I collagen. Crab protease I, which displays a distinctly different peptide bond specificity than that of crab protease II, also cleaves type I collagen as do the vertebrate metalloenzyme collagenases, which, with the exception of gelatin, do not significantly cleave noncollagenous polypeptides. In addition, the helical portion of type I collagen is not degraded by other general proteases such as chymotrypsin, elastase, thermolysin, pepsin, and papain. It is clear from these facts that the susceptibility of collagen to collagenolytic enzymes is not due solely to peptide bond specificity. The molecular interaction responsible for the susceptibility of native collagen to some proteases but not to others must, therefore, reside in that part of the enzyme which is responsible for binding substrate in a productive manner at a specific site on the macromolecular substrate. For the collagenolytic serine proteases at least, if the assumption is made that the geometry of the enzyme–substrate complex at the active site is similar for all serine proteases, which the existing evidence indicates, the protein–protein interaction that takes place during substrate binding must lead to a significant conformational change in collagen in order for peptide bond cleavage to occur. The degradation of collagen by such enzymes can, therefore, be viewed as requiring a cooperation between two specialized domains at the macromolecular interface. Recognition between these domains occurs to form the initial bimolecular complex which results in a localized denaturation of the triple helix. The ability to induce this process, which is a prerequisite to collagen degradation, distinguishes collagenases from other proteolytic enzymes. This process may involve extended enzyme–substrate interaction and may occur, at least partly, at residues significantly distant from the active site.

In this regard, the regions involved in substrate binding of the collagenolytic serine proteases may give considerable insight into the determinants of specificity in collagen degradation and may lead to a better understanding of the structural features of collagen that participate in this process.

Acknowledgments

We thank Maria L. Zapp for excellent technical assistance.

Registry No. BzValGlyArgNA, 64717-41-5; BzTyrEE, 3483-82-7; BzArgNA, 6208-93-1; collagenase, 9001-12-1; serine protease, 37259-58-8; trypsin, 9002-07-7.

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