Mode of Hydrolysis of Collagen-like Peptides by Class I and Class II *Clostridium histolyticum* Collagenases: Evidence for both Endopeptidase and Tripeptidylcarboxypeptidase Activities†

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Received April 5, 1985

ABSTRACT: The action of three class I (β, γ, and η) and three class II (δ, ϵ, and ζ) collagenases from *Clostridium histolyticum* on two series of peptides with collagen-like sequences has been examined. The peptides in the first series all contain 4-nitrophenylalanyl-Gly-Pro-Ala in subsites P_1 through P_3', but each is successively lengthened in the N-terminal direction by addition of an appropriate residue until subsite P_5 is occupied. The second group of peptides all have cinnamoyl-Leu in subsites P_2 and P_1, respectively, but each is successively lengthened in the C-terminal direction by partial additions of the Gly-Pro-Leu triplet until subsite P_6' is occupied. N-Terminal elongation causes the k_{cat}/K_M values to rise markedly and to level off after occupancy of subsite P_6 for the class I enzymes and subsite P_3 for the class II enzymes. C-Terminal elongation produces the best substrates for both classes of enzymes when subsites P_3' or P_4' are occupied by amino acids with free carboxyl groups. The k_{cat}/K_M values for the hydrolysis of both Leu–Gly bonds of cinnamoyl-Leu-Gly-Pro-Leu-Gly-Pro-Leu have been measured for both classes of enzymes. Both rates are large, but both classes preferentially hydrolyze the Leu–Gly bond of the C-terminal triplet. Thus, both classes of enzymes exhibit both endopeptidase and tripeptidylcarboxypeptidase activities.

In the previous paper (Van Wart & Steinbrink, 1985), the relative rates of hydrolysis of a series of tri-, tetra-, penta-, and hexapeptides by the class I and II collagenases[1] (EC 3.4.24.3) of *Clostridium histolyticum* were measured in order to provide a description of the sequence specificities of these enzymes. Such studies on peptides that contain a single hydrolyzable bond are an important first step in understanding the mechanism by which these collagenases hydrolyze collagen. However, in order to more closely approximate the action of these enzymes on collagen or gelatin, it is of interest to examine the mode of hydrolysis of longer substrates that contain more than one possible cleavage site. Such studies should provide insight into the pathways of hydrolysis of intact collagens by collagenases.

In this study, the action of the class I and II collagenases on two series of chromophoric peptides has been examined. The first group consists of peptides that have CNM-Leu in subsites P_2 and P_1, but each is successively lengthened in the C-terminal direction until subsite P_6' is occupied. The peptides in the second group all contain Nph-Gly-Pro-Ala in subsites P_1 through P_3', but each is successively lengthened in the N-terminal direction until subsite P_5 is occupied. Thus, each group contains peptides that can be hydrolyzed at two possible sites by the collagenases. By a combination of spectrophotometric assays and high-pressure liquid chromatographic analysis of the reaction products, the preference of the two classes of enzymes for hydrolyzing each of the two bonds has been elucidated. These studies reveal differences in the size of the active sites of the two classes of collagenases and also provide information about their mode of hydrolysis of long peptides.

Materials and Methods

Materials. All collagenases were purified as described earlier (Bond & Van Wart, 1984a; Van Wart & Steinbrink, 1985). All peptides were synthesized from intermediates prepared previously by using the dicyclohexylcarbodiimide/*N*-hydroxysuccinimide ester coupling method (Steinbrink et al., 1985). Thus, CNM-Leu-Gly-Pro-Leu-Gly-Pro-Leu was prepared by coupling CNM-Leu-Gly-Pro-Leu to Gly-Pro-Leu-OCH_3 and subsequent saponification of the ester. Peptide 6b was prepared by treating the succinimide ester of peptide 6 with NH_3. Peptides 43d and 43e were prepared by reaction of 43b and 43, respectively, with acetic anhydride (Mallinkrodt) at pH 8. The purification and characterization of all peptides were also carried out according to previously published procedures (Steinbrink et al., 1985). All peptides had the correct amino acid composition and spectral properties. High-pressure liquid chromatography grade acetonitrile was purchased from American Scientific Products and *N*,*N*-dimethyl-5-aminonaphthalene-1-sulfonyl (dansyl) chloride from Sigma Chemical Co.

Enzymatic Assays and Product Analysis. All assay were performed at 25 °C in 50 mM Tricine, 0.4 M NaCl, and 10 mM $CaCl_2$, pH 7.5. Spectrophotometric assays were monitored at 300 nm for all CNM peptides and at 308 nm for all Nph peptides using a Varian Model 219 spectrophotometer. Unless stated otherwise, all assays were carried out at substrate concentrations below K_M, and k_{cat}/K_M values were calculated as described earlier (Steinbrink et al., 1985). For peptides 55, 55a, 55b, 55c, 59, and 60, each of which has the potential to be hydrolyzed at two different sites, the rates of formation of the various products were followed by reverse-phase high-

† This work was supported by Research Grant GM27939 and Research Career Development Award AM01066 (to H.E.V.W.) from the National Institutes of Health, U.S. Public Health Service.

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[1] Abbreviations: collagenase, *Clostridium histolyticum* collagenase; FA, 2-furanacryloyl; CNM, cinnamoyl; Nph, 4-nitrophenylalanine; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; Ac, acetyl; Boc, *tert*-butyloxycarbonyl; dansyl, *N*,*N*-dimethyl-5-aminonaphthalene-1-sulfonyl.

0006-2960/85/0424-6527$01.50/0

Table I: Effect of N-Terminal Elongation on the Hydrolysis of Peptides by Class I and II Collagenases

		k_{cat}/K_M ($\times 10^{-4}$ M^{-1} min^{-1})					
	$P_6-P_5-P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'-P_4'$	β	γ	η	δ	ϵ	ζ
42c	Boc-Nph-Gly-Pro-Ala-OCH_3	<0.30	0.62	<1.8	3.3	1.6	3.7
43c	Boc-Pro	1.3	1.7	2.3	3.7	2.3	7.3
43d	Ac-Pro	11	20	12	160	95	320
49c	Boc-Gly-Pro	670	460	390	1600	790	2000
54c	Boc-Ala-Gly-Pro	630	430	420	1100	680	1300
55c	Boc-Pro-Ala-Gly-Pro	240	230	190	780	620	770
42a	Boc-Nph-Gly-Pro-Ala	8.2	5.7	4.2	35	23	52
43a	Boc-Pro	11	8.5	9.7	87	70	100
43e	Ac-Pro	150	170	150	1300	1200	1700
49a	Boc-Gly-Pro	870	660	530	1600	1400	2000
54a	Boc-Ala-Gly-Pro	2100	1600	1400	1600	1300	2100
55a	Boc-Pro-Ala-Gly-Pro	1400	1300	1000	1500	1200	2200
42b	Nph-Gly-Pro-Ala-OCH_3	<0.30	<0.20	<1.8	<1.6	<0.80	<1.1
43b	Pro	0.60	0.50	<1.8	1.7	<0.80	2.7
49b	Gly-Pro	8.7	4.6	9.4	190	150	240
54b	Ala-Gly-Pro	120	110	90	730	570	900
55b	Pro-Ala-Gly-Pro	290	280	260	660	620	940
42	Nph-Gly-Pro-Ala	<0.30	<0.20	<1.8	<1.6	<0.80	<1.1
43	Pro	11	9.3	11	100	82	170
49	Gly-Pro	820	630	550	1600	1300	1900
54	Ala-Gly-Pro	910	580	530	1600	1300	2200
55	Pro-Ala-Gly-Pro	1500	1300	1100	1800	1400	2400

pressure liquid chromatographic analysis of the reaction mixture as a function of time.

For the Nph and CNM peptides, 50-μL aliquots of the reaction mixtures were removed at various intervals and quenched with 50 μL of acetonitrile or a 0.15 mM solution of dansyl chloride in acetonitrile, respectively. The latter reagent labels products with free amino termini for subsequent fluorescence analysis. These aliquots were applied to a Rainin Microsorb 5-μm C_{18} (4.6 × 250 mm) column used with a Beckman Model 334 instrument. For the reactions with the CNM peptides 59 and 60, the products were separated with a linear gradient prepared by mixing 50 mM sodium phosphate, pH 6.5, and 50% acetonitrile in water. For the reactions with Nph peptides 55, 55a, 55b, and 55c, elutions were performed isocratically with 25%, 27.5%, 30%, and 35% acetonitrile, respectively, containing 0.1% trifluoroacetic acid. A dual detection system was employed to analyze for products. The CNM and Nph peptides were detected with a Varian Model UV-50 absorbance detector at 280 nm, while dansylated products were detected with a Gilson Spectra/Glo fluorescence detector equipped with a 330–380-nm band-pass excitation filter and a 460–600-nm band-pass emission filter.

Results

To explore the mode of hydrolysis of longer peptides with collagen-like sequences, including those that have more than one possible cleavage site, two series of chromophoric peptides have been synthesized. All of the peptides in the first group contain Nph-Gly-Pro-Ala in subsites P_1 through P_3' [nomenclature of Schecter & Berger (1967)] so that hydrolysis of the Nph–Gly bond can be monitored spectrophotometrically. The peptides in this group differ in that each has been elongated in the N-terminal direction by addition of an appropriate residue to each subsite. The rates of hydrolysis of all peptides both as blocked and free amines and as blocked and free acids have been quantitated. The second group of peptides all have CNM-Leu in subsites P_2 and P_1, respectively, but each has been elongated in the C-terminal direction by repeating portions of the Gly-Pro-Leu sequence.

The k_{cat}/K_M values for the hydrolysis of the Nph peptides by all six collagenases are listed in Table I. The first group of peptides (42c, 43c, 43d, 49c, 54c, and 55c) have both the N and C termini blocked. Peptide 42c with a Boc group in subsite P_2 is a poor substrate for both classes of enzymes. Addition of Boc-Pro to this peptide (43c) increases the rates of hydrolysis slightly. However, the increase is much larger with Ac-Pro (43d), indicating that the Boc group is sterically unfavorable in subsite P_3. Peptide 49c, which has Boc-Gly in subsites P_4 and P_3, is hydrolyzed much faster by both classes of enzymes. A further increase in the length of the peptide by addition of Boc-Ala to subsites P_5 and P_4 (54c) has little effect on the rates of hydrolysis by the class I enzymes and actually causes a small decrease for the class II enzymes. Finally, addition of Boc-Pro to subsites P_6 and P_5 causes small decreases in the rates of hydrolysis by all six enzymes.

The next set of peptides (42a, 43a, 43e, 49a, 54a, and 55a) are identical with those in the previous set, except that each is a free acid. The rates of hydrolysis of all of these peptides by all six enzymes are uniformly greater than those for the corresponding esters, though the class I enzymes prefer the free acids more strongly than the class II enzymes. As the peptide is elongated in the N-terminal direction, a large increase in the hydrolysis rates for the class I enzymes is observed on going from peptide 43a to peptides 43e and 49a. Thus, occupancy of subsite P_3 by a Boc-Gly or an Ac group greatly enhances the interaction of the peptide with these enzymes. Further elongation increases the hydrolysis rates by a more moderate amount. In contrast, the class II enzymes reach maximal activity with peptide 43e, and further elongation has little effect on the rates, indicating that interactions with subsites past P_3 are unimportant. It is also interesting to note that the hydrolysis rates of both classes of enzymes become approximately equal for peptides 54a and 55a.

The free amine derivatives of the esters (42b, 43b, 49b, 54b, and 55b) are hydrolyzed by the class I enzymes at rates that increase uniformly with increasing length of the peptide, with the largest increases observed when subsites P_3 and P_4 become occupied. In contrast, the rise to maximal activity for the class II enzymes peaks for peptide 54b. The maximal rates for both classes are approximately the same as those for the fully blocked peptide 55c. However, for the shorter peptides, both classes of enzymes prefer a blocked amino terminus.

Peptides 42, 43, 49, 54, and 55 are unblocked derivatives of the parent peptides discussed above. Peptide 42 is not

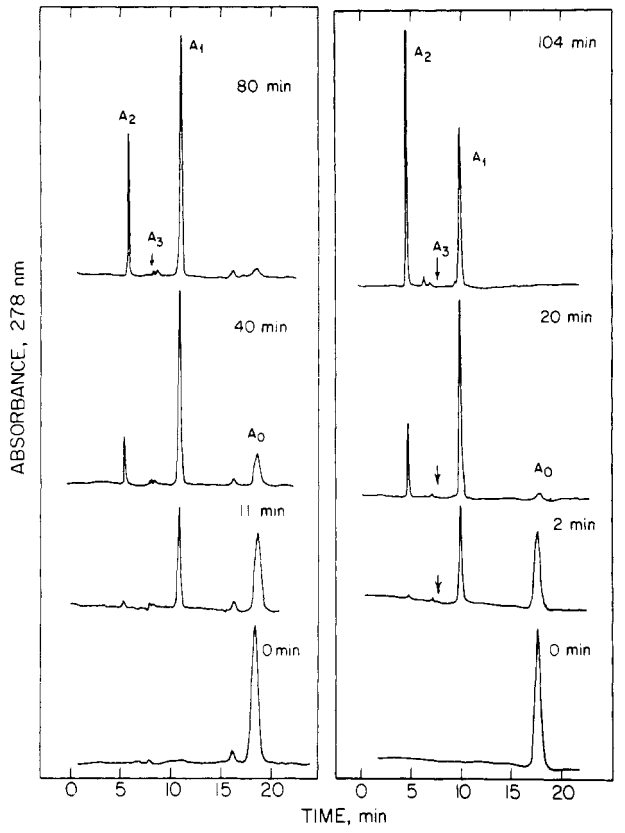
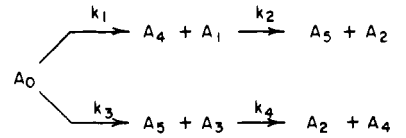


FIGURE 1: High-pressure liquid chromatographic analysis of the products (nomenclature shown in Table II) formed on reaction of peptide 55c with β-collagenase (left panel) and ϵ-collagenase (right panel). The peptides were eluted isocratically with 35% acetonitrile in water containing 0.1% trifluoroacetic acid. The substrate concentration was 50 μM for both reactions, and the concentrations of β- and ϵ-collagenases were 16 and 6.5 nM, respectively.

hydrolyzed at a detectable rate by any of the enzymes, but peptide 43 is cleaved at moderate and high rates by class I and class II enzymes, respectively. Sequential addition of amino acids in subsites P_3 through P_5 generally increases the activity of the class I enzymes, while class II enzymes show a maximum rate after Gly has been added to subsite P_3 (peptide 49). Thus, the class II enzymes do not require residues beyond subsite P_3 for rate enhancements. In contrast, the interaction of the class I enzymes with substrates extends to at least the P_5 subsite. When subsite P_5 is occupied, the hydrolysis rates of both classes toward the unblocked peptides are almost the same.

Analysis of the total digest of peptides 55–55c by thin-layer and high-pressure liquid chromatography shows that three peptides are produced. Thus, it is clear that hydrolysis is occurring at two sites, the Ala–Gly and Nph–Gly bonds. Depending on which of these bonds is hydrolyzed fastest, two mechanisms of hydrolysis are possible (Table II). The first pathway for the hydrolysis of the parent peptides, denoted A_0, involves the initial cleavage of the Nph–Gly bond to produce peptides A_1 and A_4 with subsequent hydrolysis of A_1 to produce A_2 and A_5. Alternatively, hydrolysis of the Ala–Gly bond could occur first to produce peptides A_3 and A_5, followed by the hydrolysis of A_3 to produce peptides A_2 and A_4. The k_{cat}/K_M values listed in Table I for these peptides were calculated from first-order rate constants, k_{obsd}, obtained from the absorbance changes in the assays by assuming that hydrolysis of the Nph–Gly bond of the parent peptides, A_0, was the sole event being monitored. Thus, it has been assumed that $k_1 >> k_3$, in which case $k_{obsd} = k_1$. However, there are

Table II: Possible Hydrolysis Mechanisms and Products for Peptides 55–55c (A_0)



$P_5-P_4-P_3-P_2-P_1-P_1'-P_2'-P_3'$

A_0	(Boc) ± Pro-Ala-Gly-Pro-Nph-Gly-Pro-Ala ± (OCH_2CH_3)
A_1	(Boc) ± Pro-Ala-Gly-Pro-Nph
A_2	Gly-Pro-Nph
A_3	Gly-Pro-Nph-Gly-Pro-Ala ± (OCH_2CH_3)
A_4	Gly-Pro-Ala ± (OCH_2CH_3)
A_5	(Boc) ± Pro-Ala

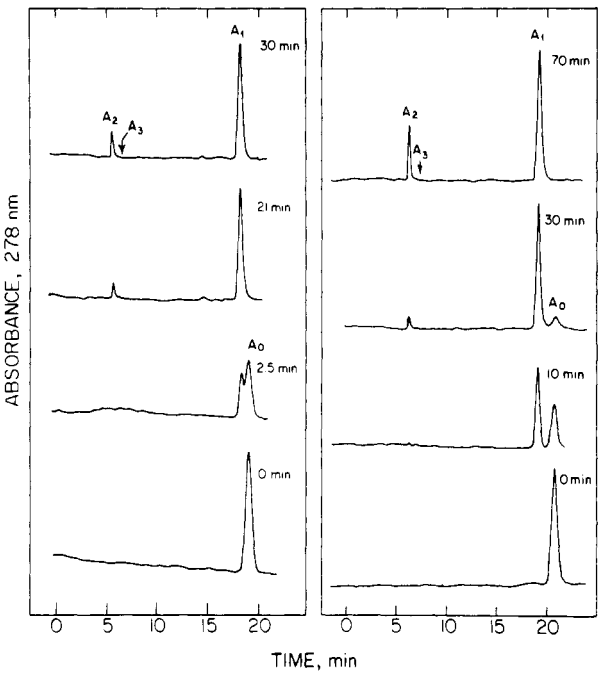


FIGURE 2: High-pressure liquid chromatographic analysis of the products (nomenclature shown in Table II) formed on reaction of peptide 55a by β-collagenase (left panel) and ϵ-collagenase (right panel). The peptides were eluted isocratically with 30% acetonitrile in water containing 0.1% trifluoroacetic acid. The substrate concentration was 50 μM, and the concentrations of β- and ϵ-collagenases were 8.2 and 6.5 nM, respectively.

two other possibilities. The second is that k_1 and k_3 are similar in magnitude, in which case $k_{obsd} = k_1 + k_3$, the sum of the rate constants for the two reactions. The third possibility, though unlikely, is that $k_3 >> k_1$ with k_{obsd} being influenced by k_3 and k_4. To measure the relative values of k_1 and k_3, the products of these reactions have been analyzed by high-pressure liquid chromatography.

Elution profiles showing the products formed during the reaction of peptide 55c by β- and ϵ-collagenases are shown in Figure 1. For both enzymes, it can been seen that the only Nph peptide that accumulates during the middle of the reaction is A_1. Peptide A_3 never appears and is apparently never produced. Thus, the rates measured correspond solely to the hydrolysis of the Nph–Gly bond of A_0, and $k_1 >> k_3$. After approximately 50% of A_0 has been converted to A_1, detectable amounts of peptide A_2, produced by the hydrolysis of A_1, start to appear. This indicates that the rates of hydrolysis of the Ala–Gly bond of A_1 by both collagenases are considerably slower than those of the Nph–Gly bond of A_0.

The same analysis has been carried out for the hydrolysis of peptide 55a (Figure 2). The elution times of some of the

Table III: Effect of C-Terminal Elongation on the Hydrolysis of Peptides by Class I and II Collagenases

		k_{cat}/K_M ($\times 10^{-4}$ M^{-1} min^{-1})					
	P_2–P_1 –P_1'–P_2'–P_3' –P_4'–P_5'–P_6'	β	γ	η	δ	ϵ	ζ
56	CNM-Leu-Gly	<0.30	<0.10	<1.1	<1.6	<0.80	<1.0
57	Gly-Pro	<0.30	<0.10	<1.1	13	5.7	12
6	Gly-Pro-Leu	160	110	110	1400	1300	1900
6b	Gly-Pro-Leu-NH_2	27	18	13	600	640	860
58	Gly-Pro-Leu-Gly	330	240	140	1800	1700	2000
59	Gly-Pro-Leu-Gly-Pro[a]	40	24	21	270	200	350
					90	70	120
60	Gly-Pro-Leu-Gly-Pro-Leu[a]	70	90	30	230	370	470
		250	310	120	440	730	930

[a] The first number listed is for hydrolysis of the first Leu–Gly bond from the amino terminus and the second for the second Leu–Gly bond of the intact substrate.

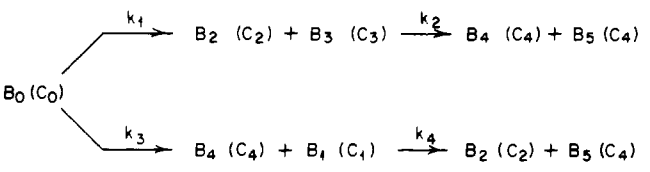
peptides differ from those in Figure 1 because the parent peptide is a free acid. However, the results are essentially the same as those for peptide 55c in that hydrolysis of A_0 first yields A_1, which is subsequently hydrolyzed to A_2. Thus, $k_1 >> k_3$ for these reactions as well. The only difference from the behavior shown in Figure 1 for peptide 55c is that no A_2 is produced until almost all of the A_0 has been hydrolyzed. This indicates that the ratio k_1/k_2 is larger for the hydrolysis of peptide 55a than 55c for both classes of collagenases. Apparently, esterification of the Ala residue in subsite P_3' lowers k_1 for peptide 55c, an effect consistent with the data presented previously (Steinbrink et al., 1985).

Similar experiments have been carried out on peptides 55 and 55b, and the elution profiles (not shown) are very similar to those for peptides 55a and 55c, respectively. In all cases, $k_1 >> k_3$, and the pathway of hydrolysis is that of A_0 first to A_1 and A_4, and next of A_1 to A_2 and A_5. Thus, the k_{cat}/K_M values listed in Table I are good estimates of the rates of hydrolysis of the Nph–Gly bond of A_0 by both classes of enzymes. With regard to the relative values of k_1 and k_2, the former is much larger for all of the peptides. However, for the esters (peptides 55b and 55c), the disparity between them is not as great as for the free acids (peptides 55 and 55a).

The mode of hydrolysis of peptides elongated in the C-terminal direction has been investigated by examining a second group of peptides, labeled 6, 6b, and 56–60. All contain CNM-Leu in subsites P_2 and P_1, but each is successively lengthened in the C-terminal direction by partial additions of the Gly-Pro-Leu triplet into the appropriate subsites. The k_{cat}/K_M values for the hydrolysis of all susceptible bonds are listed in Table III. For peptides 6, 6b, and 56–58, only the first Leu-Gly bond (i.e., that closest to the N terminus) is hydrolyzed, and the k_{cat}/K_M values listed correspond to this process. Peptide 56 is not hydrolyzed by any of the collagenases. Peptide 57, which has a Pro residue in subsite P_2', is not hydrolyzed by the class I enzymes but is hydrolyzed slowly by the class II enzymes. The rates of hydrolysis of peptide 6 by both classes of enzymes are markedly higher, reinforcing the importance of having a full triplet in subsites P_1' through P_3'. Amidation of the Leu residue in subsite P_3' lowers the rates 6–9-fold for the class I enzymes and 2-fold for the class II enzymes. This lowering is similar to that observed when the Leu residue is esterified [peptide 6a in Van Wart & Steinbrink (1985)], implying that the negatively charged carboxyl group is important. The rates for the class I enzymes are increased slightly when an additional Gly is introduced in subsite P_4', but there is almost no effect on the rates for the class II enzymes.

Peptides 59 and 60 are the first in this series to contain two hydrolyzable bonds. Two pathways of hydrolysis, shown in Table IV, are possible for these peptides. One pathway in-

Table IV: Possible Hydrolysis Mechanisms and Products for Peptides 59 (B_0) and 60 (C_0)



	P_2–P_1–P_1'–P_2'–P_3'–P_4'–P_5'–P_6'
B_0 (C_0)	CNM-Leu-Gly-Pro-Leu-Gly-Pro-(Leu)
B_1 (C_1)	CNM-Leu-Gly-Pro-Leu
B_2 (C_2)	CNM-Leu
B_3 (C_3)	Gly-Pro-Leu-Gly-Pro-(Leu)
B_4 (C_4)	Gly-Pro-(Leu)
B_5 (C_4)	Gly-Pro-Leu

volves the hydrolysis of the parent peptide (B_0 or C_0 for peptides 54 and 60, respectively) at the first Leu–Gly bond to produce CNM-Leu (B_2, C_2) and B_3 or C_3, either of which can undergo a second Leu–Gly bond cleavage. The other pathway involves hydrolysis of the second Leu–Gly bond of the parent peptide to produce B_4 or C_4 and a second chromophoric peptide (B_1, C_1), which can in turn be hydrolyzed to products. Without additional information, it is unclear which reactions are occurring and influencing the value of k_{obsd} that is calculated from the spectrophotometric assays. To determine the relative values of k_1 and k_3 for the reaction of peptides 59 and 60 with both β- and ϵ-collagenases, chromatographic analysis of the products has been carried out.

If $k_1 >> k_3$, then B_3 should accumulate during the reaction, since it should be a worse substrate than B_0. If $k_3 >> k_1$, then B_4 should be present in the early stages of the reaction. An unambiguous choice between these possibilities is readily provided by an analysis of the products of hydrolysis. The results for β-collagenases shown in Figure 3 clearly indicate that B_2 (left panel) and B_3 (right panel) are the major reaction products until most of B_0 has been hydrolyzed. B_4 and B_5 do not appear until late in the reaction and arise from the slow hydrolysis of B_3. Thus, $k_1 >> k_3$ and the k_{cat}/K_M values listed in Table III for the class I collagenases are good estimates of the rate constants for the hydrolysis of the first Leu–Gly bond of peptide 59.

Similar data for ϵ-collagenase are shown in Figure 4. It can be seen that B_1, B_2, B_3, B_4, and B_5 are all visible almost from the start of the reaction, indicating that B_0 is being hydrolyzed at both of its Leu–Gly bonds and that k_1 and k_3 are similar in magnitude. Thus, k_{obsd} calculated from the initial region of the first-order plot for this reaction is equal to $k_1 + k_3$. The k_1/k_3 ratio has been estimated from the B_2/B_4 ratio by integrating the peaks in the elution profiles. From these data, the values of k_1 and k_3 for the hydrolysis of the first and second Leu–Gly bonds, respectively, of B_0 by ϵ-collagenase

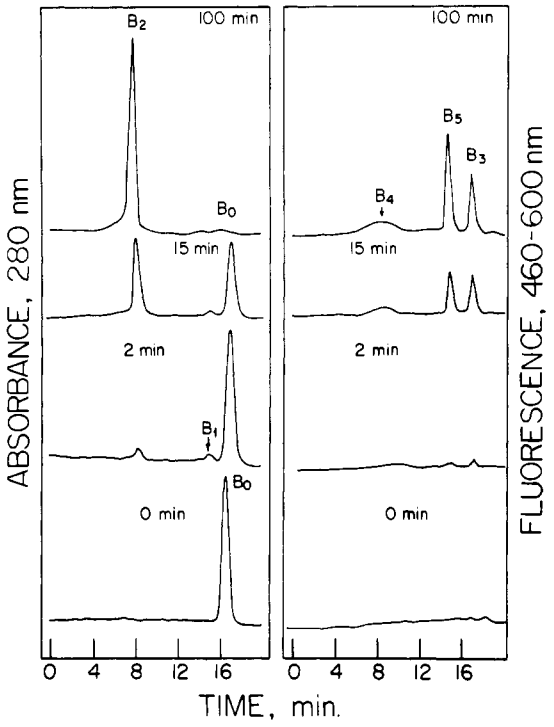


FIGURE 3: High-pressure liquid chromatographic analysis of the products (nomenclature shown in Table IV) found on reaction of peptide 59 with β-collagenase. The substrate and enzyme concentrations were 0.1 mM and 92 nM, respectively.

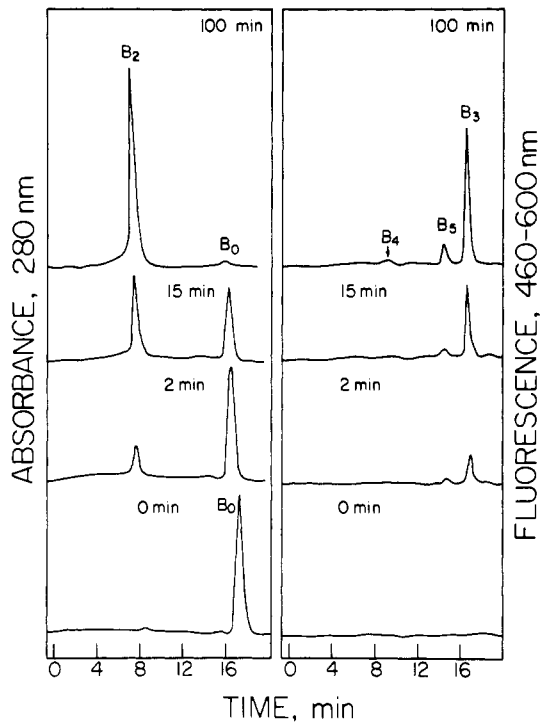


FIGURE 4: High-pressure liquid chromatographic analysis of the products (nomenclature shown in Table IV) formed on reaction of peptide 59 with ε-collagenase. The substrate and enzyme concentrations were 0.1 mM and 11 nM, respectively.

have been calculated (Table III). Values of k_1 and k_3 have also been calculated for δ- and ζ-collagenases by assuming that the k_1/k_3 ratio was the same as for ε-collagenase. It is seen that k_1 is approximately 3-fold higher than k_3. In this reaction, B_1 appears almost immediately, but its concentration remains small and almost unchanged. This indicates that k_4 must be greater than k_3. In support of this conclusion, the k_{cat}/K_M values for the hydrolysis of B_1 (CNM-Leu-Gly-Pro-Leu) by

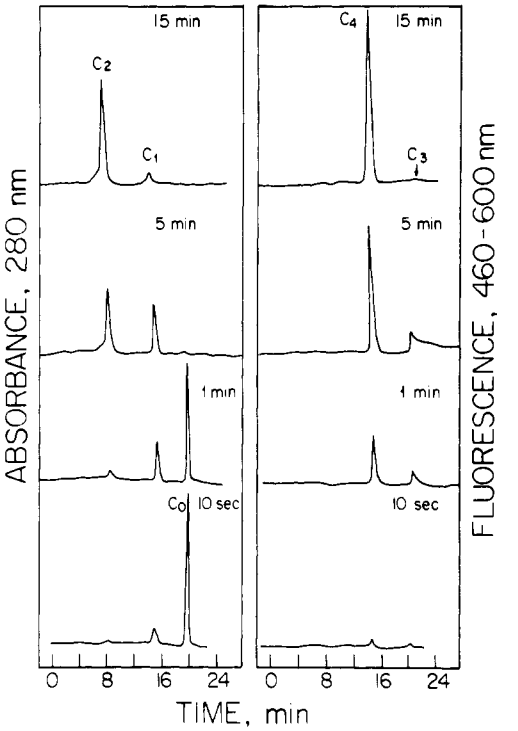


FIGURE 5: High-pressure liquid chromatographic analysis of the products (nomenclature shown in Table IV) formed on reaction of peptide 60 with β-collagenase. The substrate and enzyme concentrations were 0.1 mM and 92 μM, respectively.

the class II enzymes listed in Table III are much larger than k_3.

Similar experiments have been carried out for the hydrolysis of peptide 60 by β- and ε-collagenases. The results of the product analyses for the reaction with β-collagenase are shown in Figure 5, and it is clear that the situation is similar to that discussed above for the hydrolysis of peptide 59 by ε-collagenase. It is seen that C_1, C_2, C_3, and C_4 are all present at an early stage of the reaction. The relative values of k_1 and k_3 have been estimated from the integrated areas of the peaks corresponding to C_2 and C_1, respectively. From these data, the values of k_{cat}/K_M for the hydrolysis of both Leu–Gly bonds of peptide 60 by β-collagenase have been calculated and are listed in Table III. It is seen that k_3 is more than 3-fold larger than k_1 for this reaction.

The same analysis has been carried out for ε-collagenase. The elution profiles (not shown) are very similar to those shown in Figure 5, with the exception that the concentration of C_1 is lower than in the corresponding reaction for β-collagenase. As a result, k_3 is calculated to be only twice k_1 for this reaction. Thus, both β- and ε-collagenases exhibit a marked preference for hydrolyzing the Leu–Gly bond of the triplet closest to the C terminus.

Discussion

It is clear from previous studies that *Clostridium histolyticum* produces two classes of collagenases that have similar physicochemical properties but different sequence specificities toward short peptides (Bond & Van Wart, 1984a–c; Steinbrink et al., 1985; Van Wart & Steinbrink, 1985). The purpose of the present study was to gain a better understanding of how these collagenases hydrolyze longer collagen-like peptides that more closely approximate the natural substrates. By examining the action of both classes of enzymes on two series of peptides of different lengths, information on the size of the active sites of these enzymes has been obtained. In addition, the preference of these enzymes for hydrolyzing bonds close to or

distant from the N and C termini has been investigated.

The pattern of k_{cat}/K_M values shown in Table I for the hydrolysis of peptides elongated in the N-terminal direction points to certain differences between the two classes of enzymes. These are best seen for the two series of free acids. For the class I enzymes, the hydrolysis rates are generally enhanced as the subsites out to P_5 become occupied. Thus, the active sites of the class I enzymes must be large enough to accommodate these interactions. In contrast, elongation of the peptides past subsite P_3 does not make them better substrates for the class II enzymes, which apparently do not have binding interactions with that region of the substrate. Thus, with respect to elongation in the N-terminal direction, these collagenases act as true endopeptidases. Once the free acid substrates have been extended to have either a free or a blocked Gly residue in subsite P_3, the rates do not change appreciably. It should also be noted that the k_{cat}/K_M values for the hydrolysis of all of the longer peptides for both classes of enzymes are very similar in magnitude. It was originally thought that the class II enzymes were more active toward synthetic peptides than the class I enzymes. However, this was based on substrates containing the 2-furanacryloyl group and Leu in subsites P_2 and P_1, respectively. It can now be seen that this observation is not general and that both classes of enzymes are equally active toward longer peptides with Pro and Nph in these subsites, respectively.

The action of these collagenases on peptides elongated in the C-terminal direction is markedly different. In interpreting the rates listed in Table III, it should be kept in mind that having Leu in subsite P_1 markedly lowers the rates for the class I compared to the class II enzymes. Thus, the differences in the magnitudes of the rate constants are misleading. For both classes of enzymes, the rates increase most markedly when subsites P_1' through P_3' become occupied. Addition of Gly to subsite P_4' increases the rates slightly, but further elongation by addition of Pro to subsite P_5' or Leu to subsite P_6' causes marked decreases in the rates. Furthermore, amidation of the Leu residue in subsite P_3' also lowers the rates, particularly for the class I enzymes. This implies that these collagenases prefer to hydrolyze sequences in which there is a residue with a free carboxyl group in subsites P_3' or P_4'. We believe that elongation past subsite P_4' lowers the rates because the resultant peptides do not have the requisite free carboxyl group. However, we cannot rule out the possibility that lengthening of the peptide causes it to adopt a conformation unfavorable for binding.

The conclusion that these collagenases prefer substrates with free carboxyl groups in subsite P_3' is substantiated from the analysis of the mode of hydrolysis of peptide 60, which has two Leu–Gly bonds available for hydrolysis. The product analyses show that both classes of enzymes preferentially hydrolyze the second Leu–Gly bond, that of the C-terminal triplet. It should be noted that on the basis of the residues present in subsites P_2 and P_3, hydrolysis of the first Leu–Gly bond would have been expectd to be fastest because having CNM in subsite P_2 is extremely favorable. In contrast, when the second Leu–Gly bond is being hydrolyzed, there is Gly-Pro in subsites P_3 and P_2, respectively, which is less favorable, giving rates 3–5-fold lower [see peptides 35 and 49 in Table IV of Van Wart & Steinbrink (1985)]. In spite of this, the second Leu–Gly bond is hydrolyzed at a faster rate, underscoring the preference of both classes of enzymes for substrates with an unblocked collagen-like triplet in subsites P_1' through P_3'. The behavior of the two classes differs in two ways. First, the class II collagenases are able to hydrolyze substrates with only a dipeptide in subsites P_1' and P_2' (peptide 50). The second difference is that the class I collagenases have a more marked preference than the class II enzymes for hydrolysis of the C-terminal triplet compared to the interior Leu–Gly bond.

The two modes of hydrolysis of peptide 60 by these collagenases can be discussed in terms of endopeptidase and tripeptidylcarboxypeptidase activities. It is clear from the data presented here and elsewhere (Van Wart & Steinbrink, 1985) that both classes of collagenases hydrolyze interior bonds in collagen and peptides with the appropriate sequence and that they have true endopeptidase activity. In addition, the data presented above indicate that the enzymes, particularly the class I enzymes, have a marked tendency to hydrolyze Gly-X-Y triplets from the C-terminal end of peptides with collagen-like sequences. This is referred to as tripeptidylcarboxypeptidase activity. The data in Table III indicate that this is not a classical carboxypeptidase activity for which there is a *strict* requirement for an unblocked carboxyl group. Moreover, the enzymes will efficiently hydrolyze off a tetrapeptide as well as a tripeptide. However, with regard to their action toward collagen, gelatin, and other substrates that contain repeating Gly-X-Y sequences, it is likely that the enzymes will in fact act as tripeptidylcarboxypeptidases, at least insofar as triplets with favorable sequences are presented to the enzyme.

This combination of endopeptidase and tripeptidylcarboxypeptidase activities toward substrates with repeating Gly-X-Y triplets makes these enzymes ideally suited to be collagenases. Since native collagens have telopeptides at both ends that do not have the repeating Gly-X-Y sequence, the endopeptidase activities of these enzymes produce the initial cleavages within the triplet-helical region to expose ends with C-terminal triplets. The chains produced by these initial cleavages can next be efficiently hydrolyzed by successive tripeptidylcarboxypeptidase cleavages. This would continue until a triplet with an unfavorable sequence is encountered. It is likely that large stretches of collagen and gelatin chains can be digested by the consecutive hydrolysis of such triplets. Since the active sites of these enzymes are large, such a hydrolysis mechanism might involve translocation of the collagenases along the substrate chain immediately following hydrolysis. This could greatly facilitate the reaction by eliminating the need for dissociation and reassociation of the enzyme from the substrate before the next round of hydrolysis.

It is difficult to compare the results of this study with previous reports in the literature concerning the action of clostridial collagenases on long synthetic peptides and collagen. It is only recently that highly purified, well-defined collagenases belonging to these two classes have been prepared and characterized, and most early studies employed either crude or partially purified preparations of unknown composition. However, it is of interest to note that electron microscopic studies of the products of digestion of rat and calf skin collagens by crude collagenase at 10 °C reveal the presence of fragments of various sizes which are postulated to have arisen from digestions from the ends of the molecule (Stark & Kuhn, 1968a,b; Kuhn & Eggl, 1966). This observation may be related to the tripeptidylcarboxypeptidase activities of these collagenases found in this study. In another report, Oshima and associates (Oshima et al., 1979) have examined the action of a partially purified commerical collagenase on $(Pro\text{-}Pro\text{-}Gly)_5$. They found that the peptide was hydrolyzed with at least three apparent rates, the first being the release of the N-terminal Pro-Pro. This was followed by hydrolysis of the

resulting tridecapeptide to form a heptapeptide and a hexapeptide, which in turn were hydrolyzed to other products. These results, which would indicate that the collagenase acts preferentially at the amino-terminal end of the substrate, are in almost complete disagreement with the trends found here. It is possible that the neutral proteinase and aminopeptidase activities that contaminate most commercial preparations may be responsible for this observation.

ACKNOWLEDGMENTS

We are indebted to Lyn Kittle for her expert technical assistance.

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Isomer-Specific Proteolysis of Model Substrates: Influence That the Location of the Proline Residue Exerts on Cis/Trans Specificity

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ABSTRACT: In an effort to further develop the technique of isomer-specific proteolysis, a number of proline-containing substrates were subjected to hydrolysis in the presence of chymotrypsin, trypsin, or prolidase. The objective was to determine whether direct hydrolysis of the cis form of the substrate could occur and, if so, the extent to which it is slower than the hydrolysis of the equivalent trans form. It is shown that for both peptide and amide substrates, which contain proline at the P_2 position, the cis form can be hydrolyzed directly by either chymotrypsin or trypsin, in contrast to earlier suggestions in the literature. For similar amide substrates, it was found that chymotrypsin has a lower catalytic efficiency for the cis form, relative to the trans form, by a factor of 20 000 while, for trypsin and its substrate, the cis form was cleaved about 2000 times less efficiently. Results for a trypsin substrate with proline at the P_2' position, rather than the P_2 position, were quite different however, since there was no indication that the cis form could be directly cleaved even at the highest enzyme concentration. There was also no indication that prolidase could cleave the dipeptide Phe-Pro when the active bond itself is in the cis form. These collective results suggest that the ability of proteases to cleave a substrate with a cis peptide bond depends strongly on the location of the cis bond relative to the active bond that is being cleaved.

Proline isomerization now appears to play an exceedingly important role in the control of protein conformation (Brandts et al., 1975). Our understanding of the process has been severely restricted, however, due to the lack of an experimental technique that is capable of detecting isomerization when it occurs in proteins or large polypeptides. Recently, this laboratory has been involved in the development of the technique [for a review, see Brandts & Lin (1985)] called isomer-specific proteolysis (ISP), which takes advantage of the fact that many proteolytic enzymes show a strong preference for cleaving peptide substrates when the active bond and other bonds close to the active bond are in the trans configuration. The ISP method shows considerable promise for yielding useful information on proline isomerization, which should help to formulate a better understanding of its importance in protein structure.

Before the ISP method is used on proteins, however, a clear understanding of the specificity of proteases toward proline-containing substrates must be obtained from studies on model peptides. In examining the isomeric specificity of proteases, we have previously suggested that proline-specific aminopeptidases, such as prolidase and aminopeptidase P, will only cleave the trans form of an active X–Pro band (X being any amino acid residue). The cis form must first isomerize to the trans form before it can be cleaved (Lin & Brandts, 1979a,b, 1983a). Later, it was also shown that the configuration of an X–Pro bond located immediately adjacent to the active bond also plays an important role in peptide-bond hydrolysis. That is, trypsin can only readily cleave an active Lys–X bond in a substrate with the Lys-X-Pro sequence (i.e., proline at the P_2' position) when the following X–Pro bond is in the trans form (Lin & Brandts, 1983a,b), while proline-specific endopeptidase can only cleave an active Pro–X bond in a substrate with the -X-Pro-Y sequence (i.e., proline at P_1 position), when the preceding X–Pro bond is in the trans form (Lin & Brandts, 1983c). More recently, however, we found that chymotrypsin can rapidly cleave the Tyr–Val bond in the RNase sequence -Asn_{113}-Pro_{114}-Tyr_{115}-Val_{116}- and the Phe–Arg bond in the bradykinin sequence Arg_1-Pro_2-Pro_3-Gly_4-Phe_5-Ser_6-Pro_7-Phe_8-Arg_9 in spite of the existence of the cis form, which in these cases is two bonds away from the active bond (i.e., proline at the P_2 position; Lin & Brandts, 1984).

0006-2960/85/0424-6533$01.50/0