Synthetic Substrates of Vertebrate Collagenase

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ABSTRACT: The active site specificity of vertebrate collagenase was mapped with the synthesis of a variety of peptides, peptolides, and peptide esters. The enzyme was found to prefer very lipophilic sequences, and it was also found to be an esterase. The thio peptolide Ac-Pro-Leu-Gly-SCH[$CH_2CH(CH_3)_2$]CO-Leu-Gly-OC_2H_5 was found to be an exceptional substrate. High-performance liquid chromatography and tandem mass spectrometry were used to unambiguously establish the cleavage site in several peptide substrates.

Native vertebrate collagen is highly resistant to proteases other than vertebrate collagenase. This class of enzymes cleave vertebrate collagen at a single, specific Gly–Ile or Gly–Leu bond (Nagai et al., 1964). While the most common substrate used in collagenase assays is a ^{14}C-labeled collagen, one research group has reported the synthesis and use of small synthetic peptide substrates having amino acid sequences the same as or similar to this specific cleavage region of collagen (Nagai et al., 1976). This work was our starting place in an effort to continue mapping the active site of collagenase and to generate more sensitive substrates.

In this paper we describe a variety of peptide, peptolide, and thio peptolide substrates with a range of activity of 3–4 orders of magnitude.

Materials and Methods

Peptides **14** and **17** (see Table I) were purchased from Peninsula Laboratories, Inc. Peptides **24** and **25** were purchased from Vega Biochemicals. Other purchased chemicals were the best commercial grades available.

Synthesis. The peptides Ac-Pro-Leu-Gly-OH (**26**) and H_2N-Ile-Leu-Gly-Leu-OH (**27**) and those listed in Table I that were not obtained commercially were synthesized by the solid-phase method using generally accepted protocols (Rivier et al., 1978). The crude peptides were removed from the resin by hydrogen fluoride or ethanol–triethylamine and purified by fractional crystallization from ethanol–water or by low-pressure C_{18} reverse-phase column chromatography. The purified peptides had correct amino acid analyses and elemental analyses, consistent mass spectra, and a single peak by HPLC[1] analysis.

Synthesis of HOCH[$CH_2CH(CH_3)_2$]CO-Leu-NH_2. To a solution of L-leucine amide hydrochloride (4.6 g, 0.0275 mol) and L-α-hydroxyisocaproic acid (3.3 g, 0.025 mol) in 150 mL of dimethylformamide was added triethylamine (3.5 mL, 0.0275 mol) and HOBt (3.4 g, 0.025 mol) followed by EDC (5.3 g, 0.0275 mole). A slight exotherm was observed, and the reaction mixture was allowed to stir overnight at room temperature. The reaction mixture was filtered and the filtrate concentrated in vacuo. The residue was dissolved in ethyl acetate that was then washed with water, dilute hydrochloric acid, water, and dilute sodium bicarbonate solution and dried over magnesium sulfate. Magnesium sulfate was removed by filtration and the filtrate concentrated in vacuo yielding a solid residue, 6 g (95%) of HOCH[$CH_2CH(CH_3)_2$]CO-Leu-NH_2, which was recrystallizable from tetrahydrofuran–ethyl ether: mp 88–89 °C; NMR (Me_2SO-d_6) δ 0.89 (d, 12), 1.12–1.95 (m, 6), 3.85 (m, 1), 4.27 (m, 1), 5.43 (d, 1), 6.92 (s, 1), 7.30 (s, 1), and 7.52 (d, 1). Anal. Calcd for $C_{12}H_{24}N_2O_3$: C, 59.0; H, 9.9; N, 11.5. Found: C, 59.3; H, 10.6; N, 11.5.

Synthesis of Ac-Pro-Leu-Gly-OCH[$CH_2CH(CH_3)_2$]CO-Leu-NH_2 (**5**). To a solution of HOCH[$CH_2CH(CH_3)_2$]CO-Leu-NH_2 (0.25 g, 1.0 mmol) in 5 mL of methylene chloride was added a solution of 1,1′-carbonyldiimidazole (0.19 g, 1.2 mmol) in 5 mL of methylene chloride followed by **26** (0.39 g, 1.2 mmol). The reaction solution was stirred at room temperature for 4 days. It was then washed with water, dilute hydrochloric acid, water, and saturated sodium bicarbonate and dried over magnesium sulfate. Magnesium sulfate was removed by filtration and the filtrate concentrated in vacuo yielding a solid residue, 0.3 g (55%), a sample of which was purified by low-pressure C_{18} chromatography. The peptolide product **5** had a correct amino acid analysis, a consistent mass spectrum, and a single peak by HPLC analysis. Anal. Calcd for $C_{27}H_{47}O_7N_5$: C, 58.6; H, 8.6; N, 12.6. Found: C, 58.5; H, 8.7; N, 12.5.

The peptolides **8** and **9** were prepared in the same way.

Synthesis of Ac-Pro-Leu-Gly-$OCH_2CH_2CH(CH_3)_2$ (**21**). To a solution of **26** (0.109 g, 0.33 mmol) in 1 mL of isoamyl alcohol cooled in an ice bath was slowly added thionyl chloride (0.6 g, 0.5 mmol). The resulting solution was allowed to stand overnight at room temperature. The solution was concentrated in vacuo and the residue taken up in ethyl acetate. The ethyl acetate solution was washed with dilute sodium bicarbonate followed by several water washes and dried over magnesium sulfate. Magnesium sulfate was removed by filtration and the filtrate concentrated in vacuo yielding an oily residue that had a single peak on HPLC analysis and a mass spectrum consistent with **21**.

Synthesis of Ac-Pro-Leu-Gly-$SCH_2CH(CH_3)CH_2CH_3$ (**18**). To a stirred solution of **26** (0.33 g, 1.0 mmol) and 2-methylbutanethiol (0.42 g, 4.0 mmol) in 5 mL of methylene chloride and 2 mL of dimethylformamide was added 10 mg of 4-(dimethylamino)pyridine followed by EDC (0.23 g, 1.2 mmol). The resulting solution was allowed to stir overnight at room temperature. The solvent was removed in vacuo and the residue extracted into ethyl acetate. The ethyl acetate solution was washed with dilute hydrochloric acid, water, dilute sodium bicarbonate, and water and dried over magnesium sulfate. Magnesium sulfate was removed by filtration and the

[1] Abbreviations: HPLC, high-performance liquid chromatography; Ac, acetyl; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride; HOBt, 1-hydroxybenzotriazole; Tris, tris(hydroxymethyl)-aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; DNP, 2,4-dinitrophenyl; Z, carbobenzoxy; TFA, trifluoroacetate.

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filtrate concentrated in vacuo yielding a colorless syrup. A 100-mg sample of the crude product was purified by low-pressure C_{18} column chromatography. The purified product **18** had a correct amino acid analysis, a consistent mass spectrum, and a single peak by HPLC.

Synthesis of L-α-*Mercaptoisocaproic Acid.* L-α-Mercaptoisocaproic acid was prepared by a method reported in the literature (Yankeelov et al., 1978); $[\alpha]^{20}_D$ (ether) 31°; lit. $[\alpha]^{20}_D$ (ether) 24°.

Synthesis of $HSCH[CH_2CH(CH_3)_2]CO$-Leu-Gly-OC_2H_5. To a solution of L-α-mercaptoisocaproic acid (1 g, 6.8 mmol) and L-leucylglycine ethyl ester hydrochloride (2.0 g, 7.9 mmol) in 40 mL of dimethylformamide was added HOBt (1.1 g, 7.1 mmol) followed by triethylamine (1.1 mL, 8.0 mmol) and EDC (1.6 g, 8.3 mmol). The solution was stirred at room temperature overnight. Triethylamine hydrochloride was removed by filtration and the filtrate concentrated in vacuo. The residue was partitioned between ethyl acetate and water, and the organic layer was washed with dilute hydrochloric acid, water, and sodium bicarbonate solution adjusted to pH 8.0. The ethyl acetate solution was dried over magnesium sulfate. Magnesium sulfate was removed by filtration and the filtrate concentrated in vacuo yielding 0.86 g of syrupy product. The crude $HSCH[CH_2CH(CH_3)_2]CO$-Leu-Gly-OC_2H_5 was used in the next step without further purification.

Synthesis of Ac-Pro-Leu-Gly-$SCH[CH_2CH(CH_3)_2]CO$-Leu-Gly-OC_2H_5 (**1**). To a solution of **26** (0.7 g, 2.1 mmol), $HSCH[CH_2CH(CH_3)_2]CO$-Leu-Gly-OC_2H_5 (0.6 g, 1.7 mmol), and HOBt (0.26 g, 1.7 mmol) in 15 mL of dimethylformamide was added EDC (0.4 g, 2.1 mmol). The solution was stirred at room temperature overnight. The solvent was removed in vacuo and the residue partitioned between salt water and ethyl acetate. The ethyl acetate layer was washed with dilute sodium bicarbonate in salt water, dilute hydrochloric acid in salt water, salt water, 1.0 mM cupric sulfate in salt water (to remove any mercapto products), and finally salt water. The ethyl acetate layer was dried over magnesium sulfate. Magnesium sulfate was removed by filtration and the filtrate concentrated in vacuo yielding 0.8 g (73%) of a glassy residue of crude **1**. A 100-mg sample of this crude product was purified by low-pressure C_{18} column chromatography. The purified thio peptolide **1** had an acceptable amino acid analysis, a consistent mass spectrum, and a single peak on HPLC analysis. Anal. Calcd for $C_{31}H_{53}N_5O_8S\cdot 3H_2O$: C, 52.4; H, 8.3; N, 9.9. Found: C, 52.5; H, 7.9; N, 9.9.

Enzyme Preparation. The collagenase, prepared from normal human skin fibroblasts (Stricklin et al., 1977), was generously supplied by W. M. Moore of the Monsanto Company. The procollagenase (23 μg/mL in 0.5 M Tris and 0.01 M $CaCl_2$, pH 7.5) was activated by incubating 100-μL samples with 1 μL of trypsin (10 mg/mL in 1 mM HCl) for 20 min at room temperature followed by 20 μL of soybean trypsin inhibitor (5 mg/mL in 0.05 M Tris and 0.01 M $CaCl_2$, pH 7.5).

Kinetic Measurements. The relative rates of cleavage of the substrates listed in Table I were determined at a substrate concentration of 0.5 mM in 0.05 M Tris buffer with 0.01 M $CaCl_2$, pH 7.5, and at a temperature of 37 °C. The total reaction volume was 200 μL and contained 10–100 μL of activated enzyme. At appropriate intervals, 10-μL aliquots were withdrawn and inejcted into an Altex Model 332 HPLC equipped with a Schoeffel UV spectrophotometer Model 770 set at 220 nm and a Knauer Lichrosorb RP-18 column. The HPLC conditions were isocratic with a flow of 0.75 mL/min and a solvent composition of 20% acetonitrile and 80% water (pH 2.5, H_3PO_4). Several reaction rates could be measured at the same time by staggering the reactions by 10-min intervals and sequentially injecting aliquots for as many as seven 10-min intervals before secondary peaks interfered with the analysis. In this way, seven data points could be measured in 70 min (see Figure 1B). The relative rates reported in Table I were obtained by monitoring the appearance of the N-terminal cleavage fragment, which in 18 of the 25 substrates was **26**. Substrates that have N-terminal fragments other than **26** were allowed to react to completion. The HPLC signal for these N-terminal fragments was assumed to represent 100% reaction, and the differences in UV response between them and **26** were corrected for. The rate of cleavage of each substrate was measured simultaneously with the rate of cleavage of **4**, which was chosen as a reference and arbitrarily assigned a value of 100. Since the substrate concentrations were significantly below the K_m, pseudo-first-order kinetics were obtained and the relative initial rates for any two substrates (at constant $[E_0]$ and $[S_0]$) were proportional to the ratios of k_{cat}/K_m for the two substrates.

The data for the pH profile shown in Figure 2 were collected in the same way except the pH was varied and the spectro-

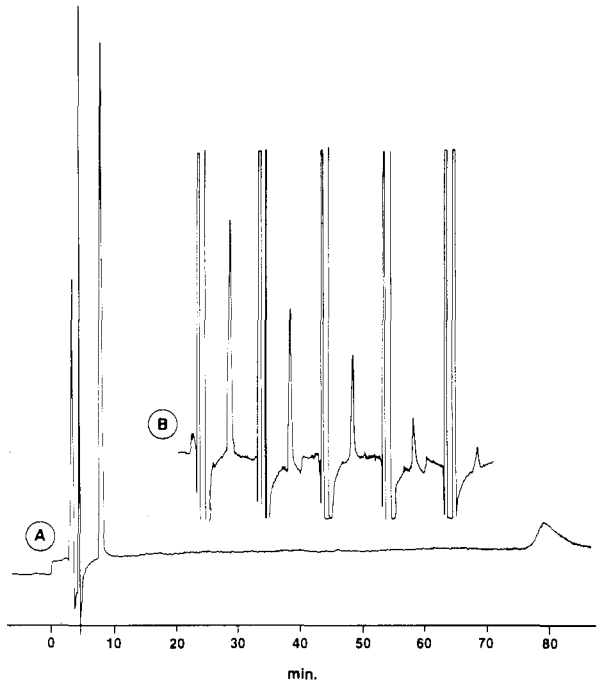

FIGURE 1: HPLC: (A) enzyme-catalyzed hydrolysis of **4**, 100% reaction; R_f (**26**), 8.0 min; R_f (**27**), 79 min; (B) correlation of initial rate of hydrolysis of **4** with concentration of enzyme. Aliquots were inserted at 10-min intervals.

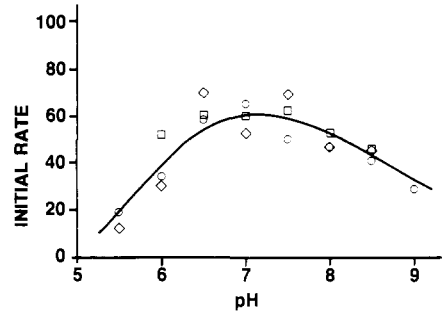

FIGURE 2: pH profiles of **1** (◇, HPLC method), **4** (□, HPLC method), and **18** (○, spectrophotometric method). Buffers used were the following: pH 5.5–6.5, MES; pH 6.5–7.5, HEPES; pH 7.5–9.0, Tris.

Table I: Synthetic Substrates

no.	substrate	rel initial rates
1	Ac-Pro-Leu-Gly-SCH[$CH_2CH(CH_3)_2$]CO-Leu-Gly-OC_2H_5	8800
2	Ac-Pro-Leu-Gly-Leu-Leu-Gly-OC_2H_5	250
3	Ac-Pro-Leu-Gly-Leu-Ala-Gly-OC_2H_5	110
4	Ac-Pro-Leu-Gly-Ile-Leu-Gly-Leu-OH	100
5	Ac-Pro-Leu-Gly-OCH[$CH_2CH(CH_3)_2$]CO-Leu-NH_2	80
6	Ac-Gly-Pro-Leu-Gly-Ile-Leu-Gly-Ala-OH	80
7	Ac-Pro-Leu-Gly-Leu-Ala-Gly-OH	66
8	Ac-Pro-Leu-Gly-OCH[$CH(CH_3)CH_2CH_3$]CO-Leu-NH_2	55
9	Ac-Pro-Leu-Gly-OCH[$CH_2CH(CH_3)_2$]CO-Ala-NH_2	52
10	Ac-Pro-Leu-Gly-Ile-Ala-Gly-OH	40
11	Ac-Pro-Leu-Gly-Ile-Leu-NH_2	38
12	Ac-Pro-Leu-Gly-Ile-Ala-Gly-Lys(TFA)-OH	30
13	Ac-Pro-Leu-Gly-Ile-Ala-Gly-Lys(TFA)-OC_2H_5	28
14	DNP-Pro-Leu-Gly-Ile-Ala-Gly-Arg-NH_2	25
15	Ac-Pro-Leu-Gly-Ile-Ala-Gly-Leu-D-Arg-OH	24
16	Ac-Gly-Pro-Leu-Gly-Ile-Ala-Gly-Ala-OH	20
17	DNP-Pro-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg-OH	5
18	Ac-Pro-Leu-Gly-$SCH_2CH(CH_3)CH_2CH_3$	5
19	Ac-Pro-Leu-Gly-Ile-Ala-OH	3.5
20	Ac-Pro-Leu-Gly-Ile-Leu-Leu-Ala-OH	3.5
21	Ac-Pro-Leu-Gly-$OCH_2CH_2CH(CH_3)_2$	2.5
22	Ac-Pro-Leu-Gly-Ile-Leu-OH	1.6
23	Ac-Leu-Leu-Gly-Ile-Leu-OH	0.2
24	Z-Gly-Leu-NH_2	0
25	Z-Gly-Ile-NH_2	0

photometric method (see below) was used for **18**. The data for the kinetic parameters reported in Table II were also collected in the same way except the concentration of substrate was varied and the spectrophotometric method was used for **1** and **18**.

The spectrophotometric assay (Weingarten & Feder, 1985) was carried out at substrate concentrations of from 0.01 to 5 mM in 0.05 M HEPES buffer with 0.01 M $CaCl_2$ at pH 6.5–7.0 and containing 0.5–1.0 mM 4,4′-dithiodipyridine. The total reaction volume was 250 μL including 10–100 μL of activated enzyme that was used undiluted with **18** and diluted 100–2000-fold with **1**. The hydrolysis was followed in microcuvettes with a Gilford Model 250 spectrophotometer, at λ = 324 nm and 25–26 °C, equipped with a Gilford Model 6051 recorder. The initial rates reported were limited to the first 5% of reaction and were corrected for nonenzymatic hydrolysis, which never exceeded 25% of the total reaction at pH 6.5–7.0. The 4,4′-dithiodipyridine was in large excess under the conditions of the initial rate assay and was found not to be rate-limiting.

Results

Synthesis. The synthesis of the peptides described in this paper was accomplished by well-established, solid-state methods. The esters and peptolides, however, required more individualized methodology. Compounds such as HOCH-[$CH_2CH(CH_2)_2$]CO-Leu-NH_2 and HSCH[CH_2CH-$(CH_3)_2$]CO-Leu-Gly-OC_2H_5 could be formed, without protecting the hydroxy or mercapto group, by coupling in the presence of HOBt and EDC. The coupling to form peptolides **5**, **8**, and **9** was carried out with 1,1′-carbonyldiimidazole, whereas the coupling to form **1** required HOBt and EDC. The ester **21** was formed by using thionyl chloride while the thio ester **18** required 4-(dimethylamino)pyridine and EDC.

Central to the synthesis of **1** was the preparation of HSC-H[$CH_2CH(CH_3)_2$]CO_2H with the same optical geometry as L-leucine. This was accomplished by the method of Yankeelov (Yankeelov et al., 1978).

Cleavage Site. The synthetic peptide analogues of collagen have been reported to cleave only at the Gly–Ile bond, but the methods used to establish the cleavage site, electrophoresis and chemical labeling, were not very sensitive. We felt it would be desirable to determine as unambiguously as possible the cleavage site; otherwise the homogeneity and even the identity of the enzyme could be in doubt. The two methods used to accomplish this were HPLC and tandem mass spectrometry (McLafferty, 1981). HPLC analysis of the cleavage products of all of the substrates having an Ac-Pro-Leu-Gly N-terminus showed a peak with retention time identical with authentic **26**. The C-terminal fragment was also generally identifiable. For **4**, our reference compound, an authentic sample of the C-terminal fragment, H_2N-Ile-Leu-Gly-Leu-OH (**27**), was prepared and found to correspond to the only other reaction product of enzyme-catalyzed hydrolysis of **4**. No other peaks were visible in the HPLC trace, suggesting the cleavage was cleanly at the Gly–Ile bond (see Figure 1A).

With tandem mass spectrometry it was possible to demonstrate that the only molecular ions visible in the spectrum of the enzyme-catalyzed reactions of **4** were consistent with **26** and **27**. Furthermore, fragmentation patterns of these ions were identical with those obtained for authentic **26** and **27**. Peptide **2** was also shown to cleave cleanly at the Gly–Leu bond by this method.

Kinetic Measurements. In earlier work the rates of enzyme-catalyzed hydrolysis of synthetic peptides were measured either by paper electrophoresis (Nagai et al., 1976) or by measuring the UV absorbance of solvent-extracted chromophoric derivatives (Masui et al., 1977). A significant methods improvement seemed to be offered by quantitative reverse-phase HPLC (Gray et al., 1981). It was possible by HPLC to monitor both the appearance of the two cleavage fragments and the disappearance of the substrate for most of the examples listed in Table I. The observation that vertebrate collagenase could catalyze the hydrolysis of esters and thio esters led to the development of a spectrophotometric assay based on thio ester **18** or thio peptolide **1** as substrates. Cleavage of these substrates yields a mercaptan that can be continuously measured in the presence of Ellman's reagent or 4,4′-dithiodipyridine (McRae et al., 1981).

The relative rates listed in Table I, the kinetic parameters listed in Table II, and the pH profile shown in Figure 2 were obtained from initial rate measurements limited to the first

Table II: Comparison of Kinetic Parameters

no.	substrate	enzyme source	K_m (M)	k_{cat} (h^{-1})	reference
1	Ac-Pro-Leu-Gly-SCH[$CH_2CH(CH_3)_2$]CO-Leu-Gly-OC_2H_5	human skin fibroblasts	3.9×10^{-3}	370 000	
2	Ac-Pro-Leu-Gly-Leu-Leu-Gly-OC_2H_5	human skin fibroblasts	1.2×10^{-3}	21 000	
4	Ac-Pro-Leu-Gly-Ile-Leu-Gly-Leu-OH	human skin fibroblasts	1.3×10^{-3}	15 500	
14	DNP-Pro-Leu-Gly-Ile-Ala-Gly-Gln-D-Arg-OH	tadpole skin	6×10^{-4}		Gray et al. (1981)
18	Ac-Pro-Leu-Gly-$SCH_2CH(CH_3)CH_2CH_3$	human skin fibroblasts	2.2×10^{-2}	2 300	
	vertebrate collagen	human skin fibroblasts	$(0.7–2.1) \times 10^{-6}$	1–565	Welgus et al. (1981)
	vertebrate collagen	rheumatoid synovial cells	2.6×10^{-6}		Harris & Vater (1980)
	vertebrate collagen	mouse bone explants	6×10^{-6}		Vaes (1971)

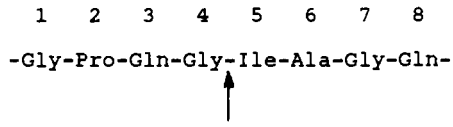

FIGURE 3: Cleavage region of α1-CB7 chick skin collagen (Highberger et al., 1978). Arrow indicates cleavage.

5% of reaction. The rates were shown to be linear over the first 10% of reaction and proportional to enzyme concentration.

Figure 2 shows the correlation of enzyme activity with pH for three substrates, and although the enzyme concentrations and temperatures differ, the initial rates are arbitrarily plotted on the same scale. The data indicate a pH optimum of 6.5–7.5, which is about 1 pH unit lower than values reported for tadpole skin collagenase (Hori & Nagai, 1979) and pig synovial collagenase (Tyler & Cawston, 1980).

Discussion

Vertebrate collagenases cleave native vertebrate collagens at a single specific Gly–Ile or Gly–Leu bond located three-fourths of the distance from the N-terminus (Harris & Krane, 1974). The known amino acid sequences of this cleavage region are rational starting points for designing synthetic substrates. Figure 3 shows a typical cleavage region, and it was just such a sequence Nagai and co-workers began to modify. They reported the minimum-size peptide substrate to be a pentapeptide (positions 2–6) and proline at position 2 to be essential to activity. Also, replacement of glutamine at position 3 by a leucine produced a more reactive substrate (Masui et al., 1977), and a leucine at position 5 yielded a more active substrate than one with isoleucine (Sakakibara et al., 1977). If one keeps in mind that Nagai used enzyme derived from tadpole skin and we used enzyme derived from human skin, a look at Table I indicates good agreement. Nagai's conclusion that proline is essential in position 2 was based on a comparison of two peptides, one with a proline at position 2 and another terminated at position 3. We prepared peptides with other residues at position 2 and found proline to be preferred but not absolutely required (**22**, **23**; Table I). Just as Nagai demonstrated, the replacement of glutamine at position 3 by leucine increased hydrolytic activity; we showed the replacement of alanine at position 6 by leucine also increased activity (**2**, **3**). Some collagens normally have a leucine in this position (Dixit et al., 1979). It appears as though the preferred cleavage sequence is extremely lipophilic.

A large rate enhancement is observed in going from pentapeptide **19** to hexapeptide **10**. Most of the enhancement may be due to displacing the ionic carboxyl group away from the cleavage site since a similar enhancement is observed in going from pentapeptide **22**, terminated by a free carboxyl, to pentapeptide **11**, terminated by a neutral amide. Examination of substrates **6** and **12–16** suggests addition of amino acid residues below position 2 or above position 7 has only a secondary influence. A 20-residue peptide, duplicating the sequence of a native collagen, with 10 residues on either side of the cleavage site, has been reported to be no more active than the smaller synthetic peptides (Gross et al., 1980).

Interestingly, when glycine of position 7 is replaced by a leucine, activity declines sharply (**4**, **20**). Apparently the wrong residue is as bad as no residue. Perhaps this is not too surprising since the native collagen polymer is a repeating Gly-X-Y.

Although esters are generally more sensitive to hydrolysis than are amides (*Tables of Chemical Kinetics, Homogeneous Reactions*, 1956), esters have not been reported as substrates for vertebrate collagenase. The most novel results shown in Table I is that vertebrate collagenase can catalyze the hydrolysis of esters. **21** was the first ester we examined in which the enzyme-catalyzed hydrolysis was convincingly above background hydrolysis. It, however, was a poor substrate. Therefore, peptolides **5**, **8**, and **9** were synthesized, and a 20–30-fold increase in activity was observed. Although the peptolides are substantially more active as substrates than the simple ester, they are only marginally better than the isosteric peptides (**8**, **11**). We expected thio esters to be even more hydrolytically sensitive than oxygen esters, so **18** was prepared. When **18** was shown to be a substrate, the possibility of active substrates whose hydrolysis could be monitored spectrophotometrically was realized. The thio peptolide **1**, which is isosteric with our most active peptide, **2**, was then prepared and found to be an exceptional substrate, more than 30 times as active peptide **2** and almost 2000 times as active as the thio ester **18**.

The enzyme-catalyzed hydrolysis of **1**, **2**, **4**, and **18** followed Michaelis–Menten kinetics, and a K_m and a k_{cat} were obtained for each from Lineweaver–Burk plots. Table II summarizes kinetic data from several sources for the hydrolysis of synthetic and native substrates catalyzed by various collagenases. All of the native substrates bind considerably more strongly to the enzyme than do the synthetic substrates. The K_m values for **1** and **2** are similar. Apparently, substitution of a sulfur atom for an amide nitrogen, –NH–, does not greatly modify binding, although the rate of cleavage increases significantly with this change. Peptide thio ester **18**, missing the amino acid residues to the right of the cleavage site, shows diminished binding. Thio ester **18** can also be used as a substrate for a spectrophotometric assay.

In conclusion, we have reported a varieity of substrates demonstrating the enzyme's preference for very lipophilic sequences as well as its activity as an esterase. Hopefully, several of these novel substrates will find use in improved assays for vertebrate collagenase.

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