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MODE OF ACTION OF BACTERIAL COLLAGENASE ON A SYNTHETIC SUBSTRATE, (Pro-Pro-Gly)$_5$

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Summary

Clostridial collagenase (EC 3.4.24.3) catalyzes the hydrolysis of (Pro-Pro-Gly)$_5$ at a minimum of three different rates, producing Pro-Pro, Gly-Pro-Pro and Gly-Pro-Pro-Gly, and various intermediate peptides. The intermediate and final products were separated by cation-exchange column chromatography and identified, and their rates of formation were measured.

Pro-Pro was released most rapidly with formation of the tridecapeptide. After the initial release of the N-terminal Pro-Pro, hexa- and heptapeptides were formed in larger amounts than tri-, tetra-, nona- and decapeptides from the tridecapeptide. The rates of disappearance of the intermediates decreased in the order trideca- > deca- and nona- > heptapeptide. The results indicate that the enzyme hydrolyzes inner linkages of the tridecapeptide having N- and C-terminal Gly residues, forming large peptides, preferentially to outer linkages, forming the tri- and tetrapeptides.

Introduction

Seifter and Harper [1] reviewed the specificity of Clostridial collagenase, but its action is still not clearly understood. The enzyme hydrolyzes many synthetic substrates of small molecular weight, and produces a mixture of peptides with molecular weights of 500—800 from collagen, depending on the source of the substrate. The enzyme seems to act as an exopeptidase, hydrolyzing the collagen stepwise from the N- or C-terminal to produce mainly tripeptides. However, it may also act as an endopeptidase, cleaving inner parts of the substrate at random to form larger fragments than tripeptides. With gelatin as substrate, it converts only 25% by weight to the two tripeptides,

Gly-Pro-Hyp and Gly-Pro-Ala [2], the other products presumably being peptides of higher molecular weight. Several of these peptides have been isolated from enzyme digests of gelatin and their sequences have been determined [3]. Surprinsingly, most of these peptides were found to contain sequences that are susceptible to the enzyme, and it is unknown why the were not degraded further.

To obtain more information on the mechanism of action of Clostridial collagenase, we have examined the kinetics of action on the synthetic substrate, $(Pro\text{-}Pro\text{-}Gly)_5$, separating the intermediates by column chromatography.

Experimental

Materials. Gly-Pro-Pro and Pro-Pro were obtained from Vega-Fox Biochemicals (Tucson, AZ, U.S.A.). $(Pro\text{-}Pro\text{-}Gly)_5 \cdot 5\,H_2O$ was from the Protein Research Foundation (Osaka, Japan). A collagenase (1000 Mandl units/mg) from *Clostridium histolyticum* was purchased from Seikagakukogyo (Tokyo, Japan). Gly-Pro-Pro-Gly-Ala-Hyp (R_F 6 of F-II), Gly-Pro-Hyp-Gly-Ala-Ile-Gly-Pro-(Ser, Gly, Pro, and Ala) (R_F 7 of F-II) and Gly-Pro-Ile-Gly-Ser-Val-Gly-Ala-Hyp (R_F 9 of F-II) were isolated from gelatin digests as descirbed previously [3].

Enzyme assay. Unless otherwise mentioned, the reaction mixture, containing 1 μmol $(Pro\text{-}Pro\text{-}Gly)_5$, 0.1 mmol Tris-HCl (pH 7.5), 1 μmol $CaCl_2$ and 0.86 μg enzyme in 1 ml, was incubated at 37°C for an appropriate period, and then 1 ml 0.2 N citric acid was added. The amino groups released were assayed by the ninhydrin method [4] with Gly-Gly as standard. Peptides were separated and determined as described below with Gly-Gly and Pro-Pro as standards.

Ion-exchange column chromatography. A JEOL amino acid analyzer 5 AH was used. The digest was applied to a Dowex 50X2 (200—400 mesh) column (0.8 X 65 cm) and eluted with 0.2 N sodium citrate buffer first of pH 3.30 and then pH 4.25 at 60°C. A part of the eluate was taken for ninhydrin assay and fractions of the remaining eluate were collected at 2.5-min intervals.

Properties of isolated peptide fragments. Gly-Pro-Pro and Pro-Pro in the digest were identified by comparison of elution times with those of authentic samples chromatographed under the same conditions. They were eluted after 275 and 230 min, respectively. The 5 other fractions shown in Fig. 2 were collected in repeated chromatographic steps. The respective fractions were combined, concentrated by evaporation and desalted by gel filtration on a Sephadex G-25 column (2.5 X 80 cm). Peptides could be detected in the concentrated fractions by the ninhydrin spot test. Fraction V was further separated on JEOL LC-R-1 resin under the same conditions as those described for Dowex 50X2. The unknown peptide and Pro-Pro were eluted after 115 and 200 min, respectively. The concentrated fraction was again desalted on Sephadex G-25. For determination of the apparent molecular weights of the peptides samples of the 5 desalted fractions were applied to a Sephadex G-25 column (1.5 X 90 cm) in a JEOL analyzer and eluted with 0.2 N sodium citrate buffer (pH 3.30) at a flow rate of 0.53 ml/min at room temperature. The V/V_0 values of the standard materials, R_F 7 or F-II, R_F 9 of F-II, R_F 6 of F-II, Gly-Pro-Pro and Pro-Pro, were 1.41, 1.52, 1.72, 1.96 and 2.04, respectively. V_0

was determined with blue dextran 2000.

A sample of each fraction was hydrolyzed with 6 M HCl for 24 h at 110°C and then the ratio of proline to glycine in the hydrolyzate was determined in the analyzer.

Results

The Clostridial collagenase hydrolyzed 44.7 μmol gelatin (Merck) per min per mg enzyme with concentrations up to 3.2 mg gelatin per incubation. Analysis with ninhydrin showed that the amount of amino groups liberated from $(Pro\text{-}Pro\text{-}Gly)_5$ by the collagenase increased with time of incubation, indicating that $(Pro\text{-}Pro\text{-}Gly)_5$ acted as a substrate (Fig. 1). It was hydrolyzed at a minimum of two apparent rates, first fast and then slow, at rates of 30.3 and 3.2 μmol/min per mg, respectively. The fast rate was about two-thirds of the rate of hydrolysis of the natural substrate gelatin. $(Pro\text{-}Pro\text{-}Gly)_5$ has four sites that should be susceptible to the enzyme, and the observed rates may reflect the different susceptibilities of these sites.

A homologous series of oligomeric peptides (Plastein) has been separated effectively by gel filtration [5], but the digestion products of $(Pro\text{-}Pro\text{-}Gly)_5$ could not be clearly separated by Sephadex G-25 gel filtration, owing to diffusion during the procedure. Therefore we separated them by cation exchange column chromatography on Dowex 50X2. Although only three peptide, Pro-Pro, Gly-Pro-Pro and Gly-Pro-Pro-Gly, should be obtained after extensive incubation of the substrate with the enzyme, we obtained several peaks of peptides after 6 or 120 min digestion (Fig. 2). Thus, the enzyme did not hydrolyze a specific bond selectively, but hydrolyzed several bonds

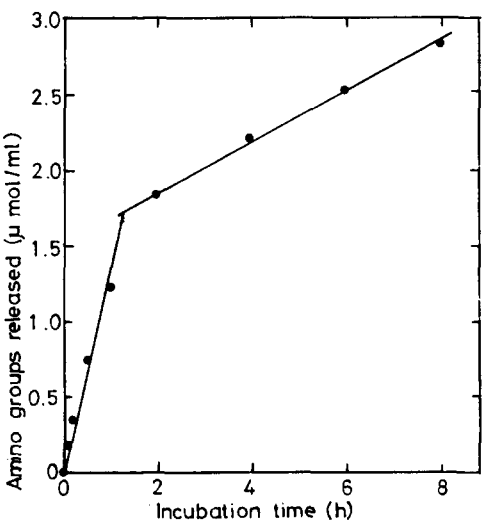

Fig. 1. Hydrolysis of $(Pro\text{-}Pro\text{-}Gly)_5$ by Clostridial collagenase. The reaction mixture contained 1 μmol $(Pro\text{-}Pro\text{-}Gly)_5$ 0.1 mmol Tris-HCl (pH 7.5), 1 μmol $CaCl_2$ and 1 μg enzyme in 1 ml and was incubated at 37°C. The reaction was stopped with 1 ml 0.2 N citric acid after various times and aliquots (0.2 ml) were assayed by the ninhydrin method with Gly-Gly as standard.

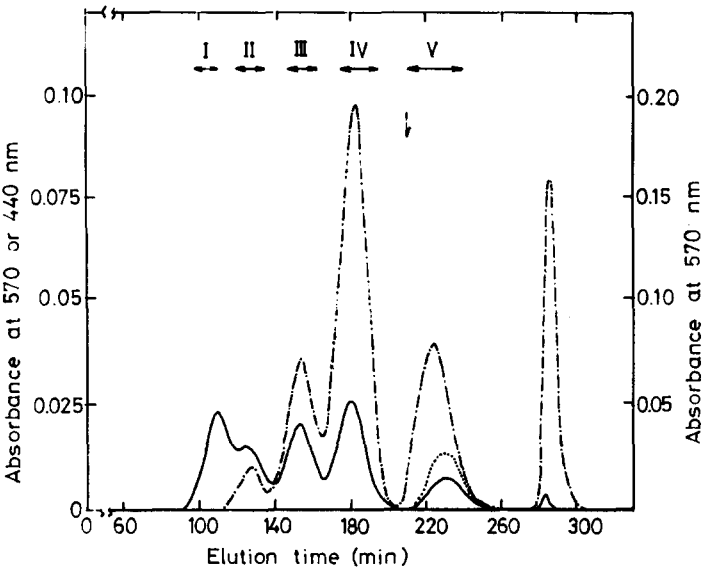

Fig. 2. Column chromatograms of the digests of $(Pro\text{-}Pro\text{-}Gly)_5$ on Dowex 50-X2. Samples prepared as in Fig. 1 using 0.86 μg enzyme were applied to a Dowex 50-X2 column (0.8 × 65 cm). The column was eluted with 0.2 N sodium citrate buffer, first at pH 3.30 and then at pH 4.25, (arrow) at 60°C and 0.83 ml/min flow rate. Digest in 6 min: ———, absorbance at 570 nm; · · · · · ·, color with ninhydrin at 440 nm (both left scale). Digest in 120 min: · — · — ·, absorbance at 570 nm (right scale). Fractions indicated by ↔ were collected.

simultaneously, producing several peptides. The peak in Fig. 2 indicated by a dotted line (recorded as color intensity with ninhydrin at 440 nm) was identified as Pro-Pro. Similarly, the last peak eluted was identified as Gly-Pro-Pro. The other fractions indicated as I—V in Fig. 2 were collected. The respective fractions obtained in repeated chromatographic steps were combined, concentrated and desalted. Since fraction V overlapped Pro-Pro, it was purified by further chromatography on JEOL LC-R-1 resin. The apparent molecular weight and Pro/Gly ratio of each peptide fraction were determined (Table I). The Pro/Gly ratio of fraction II differed from those of either the nona- or decapeptide. When this fraction was rechromatographed on LC-R-1 resin, two incompletely separated peaks were observed after 55 and 60 min, indicating that fraction II was a mixture of two peptides. The data in Table I suggest that fractions I—V are the tridecapeptide, a mixture of the deca- and nonapeptides, the hepta-, hexa- and tetrapeptide, respectively.

The time course of formation of these peptides from $(Pro\text{-}Pro\text{-}Gly)_5$ by the enzyme were examined. Pro-Pro was released almost instantaneously from the N-terminal of the substrate with production of the tridecapeptide (Fig. 3). Though this tridecapeptide was then rapidly cleaved by the enzyme, it was produced at a maximum of only 0.077 μmol Gly-Gly equivalent/μmol substrate. Although Gly-Pro-Pro has the same molar absorbance intensity with ninhydrin as Gly-Gly, the tridecapeptide may have a lower molar absorbance intensity than that of the standard. Taking the value extrapolated to zero times as 1 μmol, we calculated the apparent initial rate of overall hydrolysis of the

TABLE I

PROPERTIES OF PEPTIDES FORMED FROM (Pro-Pro-Gly)$_5$ BY CLOSTRIDIAL COLLAGENASE

Fraction	Molecular weight		Ratio of proline to glycine		Proposed sequence [d]	No. of residues
	Observed [a]	Calculated [b]	Observed [c]	Calculated [b]		
I	1100	1044	1.60	1.60	GPPGPPGPPGPPG	13
II	760	747 [e]	1.65	1.71 [e]	GPPGPPGPPG and GPPGPPGPP	10 and 9
III	575	577	1.41	1.33	GPPGPPG	7
IV	537	520	1.93	2.00	GPPGPP	6
V	302	326	1.08	1.00	GPPG	4

[a] Determined by Sephadex G-25 gel filtration.
[b] Calculated from sequence.
[c] Determined by amino acid analysis taking glycine as 1.0.
[d] G, Gly and P, Pro.
[e] Assuming equal molar amounts for nona- and decapeptides.

tridecapeptide by the enzyme as approx. 20 μmol/min per mg. The tri- and tetrapeptides were formed by cleavage of the Pro^3-Gly^4 and Pro^9-Gly^{10} bonds of the tridecapeptides with production of the deca- and nonapeptides, respectively. After 34 min digestion, the reaction mixture contained 0.12 μmol of the nona- plus decapeptides, 0.054 μmol of the tripeptide and 0.057 μmol of the tetrapeptide. Since the sum of the amount of tri- and tetrapeptides was similar

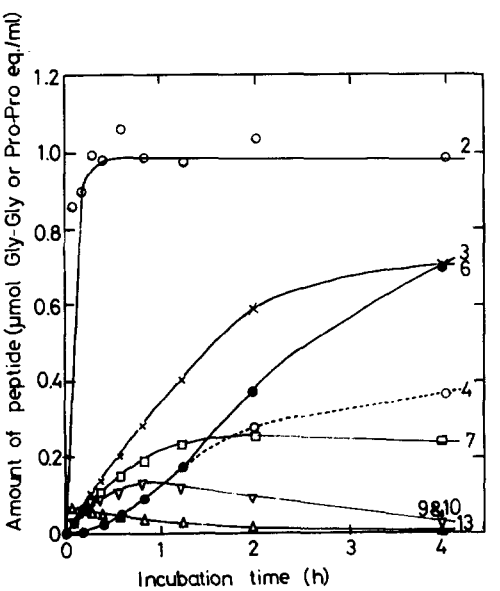

Fig. 3. Time course of peptide formation from the substrate. Digests were separated as in Fig. 2, and the amount of peptide was measured from the peak area with ninhydrin using Gly-Gly or Pro-Pro as standards. Figures inserted on the right side indicate the number of amino acids in the peptide. Since tridecapeptide had a lower molar absorbance intensity than Gly-Gly, it gave a low content in the Figure. (○———○) Pro-Pro; (●———●), tripeptide; (○· · · · ·○), tetrapeptide; (X———X), hexapeptide; (□———□), heptapeptide; (▽———▽), a mixture of nona- and decapeptide; (△———△), tridecapeptide.

to that of the mixture of nona- and decapeptides, the molar absorbance intensity of the nona- and decapeptides with ninhydrin may be identical with that of Gly-Gly. The nona- and decapeptides seem to be apparently formed at almost the same rates from the tridecapeptide, judging from the amounts of the tri- and tetrapeptides formed during the initial period of digestion. The tridecapeptide contains an additional susceptible bond and can be hydrolyzed by the enzyme to the hexa- and heptapeptides. The apparent rates of formation of the hexa- and heptapeptides were faster than those of the tri- and tetra- or the deca- and nonapeptides during the initial period of digestion by the enzyme. These facts suggested that of the three susceptible bonds in the tridecapeptide the enzyme preferentially hydrolyzes those in the center of the molecule.

The intermediate peptide formed by the enzyme were hydrolyzed further to smaller peptides. The rates of cleavage of the intermediate peptides decreased in the order of tridecapeptide > a mixture of deca- and nonapeptides > heptapeptide. This shows that the enzyme hydrolyzes large peptides more rapidly than smaller ones, and supports the idea that it preferentially hydrolyzes inner bonds of substrate molecules.

Formation of tri- and tetrapeptides was slow during the initial period of digestion, and then increased exponentially (Fig. 3). These findings are also consistent with the idea that the enzyme first hydrolyzes inner bonds of tridecapeptide to form larger peptides and then, after consumption of the molecules with inner bonds, it hydrolyzes the outer bonds of the resulting smaller peptides to form the tripeptide. Since extensive hydrolysis of 1 mol $(Pro\text{-}Pro\text{-}Gly)_5$ should finally yield 1 mol dipeptide, 3 mol tripeptide and 1 mol tetrapeptide, the rates of formation of the tri- and tetrapeptides differed from each other in the exponential phase. However, after 4 h digestion the tripeptide was formed only twice as fast as the tetrapeptide. This indicates that the enzyme hydrolyzes the Pro-Gly bond, 4 amino acyl residues from the C-terminal, slightly more readily than when the distance is 3 amino acyl residues from the N-terminal. This conclusion is supported by the finding that later the heptapeptide was hydrolyzed slightly at a time when the hexapeptide was still not cleaved. A total of 2.01 μmol peptide other than Pro-Pro was produced from 1 μmol $(Pro\text{-}Pro\text{-}Gly)_5$ after 4 h digestion by the enzyme, indicating that about half the susceptible bonds in the substrate were hydrolyzed.

The apparent initial rates of hydrolysis of the four susceptible bonds of $(Pro\text{-}Pro\text{-}Gly)_5$ were calculated (Table II). Since, except for Pro-Pro and the tri- and tetrapeptides, all the peptides should be intermediates, their rates of formation may indicate their overall rates of increase (formation minus decomposition), rather than their absolute rates of formation. N-Terminal Pro-Pro was most rapidly released from the substrate with formation of the tridecapeptide. However, the rate of Pro-Pro formation was far from the true initial velocity, because 90% was hydrolyzed within 6 min. Since the error in measurement was rather large and the molar absorbance of Pro-Pro with ninhydrin is only about 5% that of Gly-Gly, no further attempts were made to determine the actual initial rate. Of the three susceptible sites in the tridecapeptide, the enzyme hydrolyzed the one inner bond about 3 times faster than the two outer ones, again indicating that it acted as an endopeptidase rather than an exopeptidase.

TABLE II

APPARENT INITIAL RATES OF HYDROLYSIS OF THE FOUR SUSCEPTIBLE BONDS OF (Pro-Pro-Gly)$_5$ BY THE COLLAGENASE

Bonds cleaved	Rate (μmol/min per mg)
Pro-Pro↓Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro-Pro-Gly	168 [a]
Gly-Pro-Pro↓Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro-Pro-Gly	2.17 [b]
Gly-Pro-Pro-Gly-Pro-Pro↓Gly-Pro-Pro-Gly-Pro-Pro-Gly	6.20 [c]
Gly-Pro-Pro-Gly-Pro-Pro-Gly-Pro-Pro↓Gly-Pro-Pro-Gly	2.29 [b]

[a] Calculated from Pro-Pro formation. The rate of Pro-Pro formation was far from the true initial velocity, because 90% was hydrolyzed within 6 min.

[b] Calculated from the overall rates of formation of nona- and decapeptides and ratio of Gly-Pro-Pro to Gly-Pro-Pro-Gly formed initially. The last two peptides were in part derived from hexa-, nona- and decapeptides formed once.

[c] Calculated from the rate of heptapeptide formation.

The elution times of the peptides are plotted against their log molecular weight in Fig. 4. The almost instantaneous release of the N-terminal Pro-Pro reduced the number of possible intermediate peptides considerably. The order of elution of the peptides from a Dowex 50X2 column was trideca-, deca- and nona-, hepta-, hexa- and tetrapeptides, suggesting that the peptides were separated by the molecular sieving action of the resin. On the other hand, their

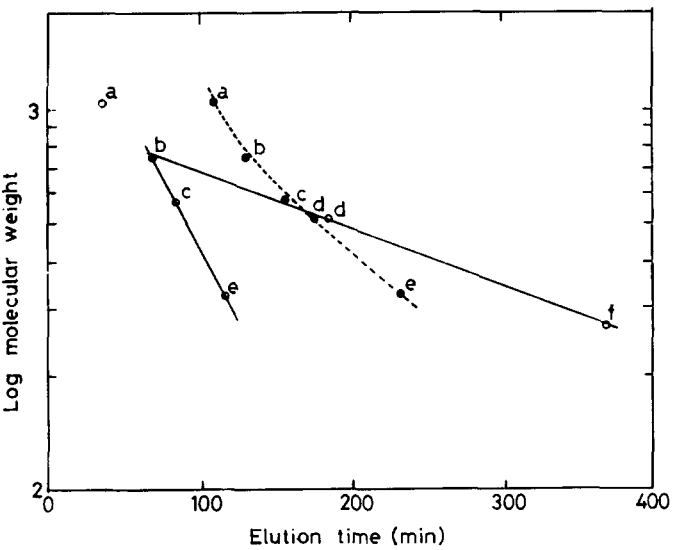

Fig. 4. Relationship between peptide size and elution time from a cation-exchange column. Peptides were chromatographed on Dowex 50-X2 (●· · · · · ●) or JEOL LC-R-1 resin (○———○) columns with 0.2 M sodium citrate buffer (pH 3.30) at 60°C and 0.83 ml/min flow rate. Elution times are plotted against the log molecular weights of the peptides. a, tridecapeptide; b, average for deca- and nonapeptide; c, heptapeptide; d, hexapeptide; e, tetrapeptide; f, tripeptide.

order of elution from JEOL resin reflected their structure. The peptides could be separated into two groups: one of oligomeric forms of the triad, Gly-Pro-Pro, and the other of peptides with C-terminal glycine. The peptides in each group were eluted from the column in the order of large to small peptides. These findings strongly support our previous conclusion that amino acid homo-oligopeptides are separated by the molecular sieving action of LC-R-1 resin [6].

Discussion

It has been found that Hyp-Gly-Gly-Pro and carbobenzoxy-Pro-Gly-Gly-Pro do not act as substrates for Clostridial collagenase, whereas the methyl ester of the latter does [7]. Moreover, the hexapeptides isolated from digests of gelatin by the enzyme contain sequences that are thought to be susceptible to the enzyme [3], but that proved resistant to further degradation when incubated with the enzyme at the same ratio by weight of enzyme to peptide as that used to hydrolyze geltain (Oshima, G., Shimabukuro, H. and Nagasawa, K., unpublished data). Furthermore, it has been shown that a hexapeptide blocked at the N-terminal is faster hydrolyzed by the enzyme than the free hexapeptide [8]. These findings suggest that charged groups on the N- and C-terminals of hexapeptides inhibit the cleavage of susceptible bonds.

In this work we found that the enzyme hydrolyzed $(Pro\text{-}Pro\text{-}Gly)_5$ at least two apparent rates, first fast and then at about 1/10 of the initial rate. The results in Fig. 3 indicate that the slow reaction represented mainly formation of the tripeptide, Gly-Pro-Pro. Many peptides were formed during the reaction (i.e., trideca-, deca-, nona-, hepta-, hexa-, tetra-, tri- and dipeptides), but the dipeptide, Pro-Pro, was released much faster and more specifically than the other peptides. The rapid release of Pro-Pro indicates that a free N-terminal imino group of the substrate does not affect any catalytic action of the enzyme. Thus it is speculated from our results that the ester or amide form of Hyp-Gly-Gly-Pro and Pro-Gly-Gly-Pro as well as Pro-Pro-Gly-Pro may serve as synthetic substrates for the enzyme.

The hepta- and hexapeptides were formed about 3 times faster than the tri- and tetrapeptides from the tridecapeptide during the initial phase of digestion. Thus a free amino or carboxyl group, 3 or 4 amino acy residues from a susceptible bond, seems to influence the rate of its hydrolysis. Since the tridecapeptide was almost completely hydrolyzed within 2 h, the tripeptide was presumably formed from hexa-, hepta-, nona- and decapeptides. The enzyme produced tri- and tetrapeptides equally readily by a single cleavage of the deca- and heptapeptides, and formed two tri- and one hexapeptides from the nonapeptide. Almost equal amounts of nona- and decapeptides were produced during initial phase of digestion, and a mixture of these peptides should yield three tri- and one tetrapeptides. The amount of tripeptide formed from the hexapeptide in the period of 2—4 h of digestion was calculated by substracting 3 times the amount of the tetrapeptide formed and the amount of heptapeptide disappearing from the total amount of tripeptide formed. The rate of formation of the tripeptide from hexapeptide was calculated to be 0.5 μmol/min per mg, which is only about 1/60 of the initial rate of hydrolysis of $(Pro\text{-}Pro\text{-}Gly)_5$ by the enzyme. This slow rate indicates that the free amino and

carboxyl groups, 3 amino acyl residues from the susceptibility bond in the hexapeptide, inhibit the hydrolysis of this bond by the enzyme.

If the enzyme acts as an exopeptidase, catalyzing the stepwise hydrolysis of the N- or C-terminal part of collagen or gelatin, it should produce mainly tripeptides. However, if it acts as an endopeptidase, hydrolyzing the substrate at random to form hexapeptides, it would produce tripeptides slowly from the hexapeptides, because the amino and carboxyl groups at the ends of the hexapeptide would inhibit its further hydrolysis. Although the enzyme serves at first on $(Pro\text{-}Pro\text{-}Gly)_5$ as an exopeptidase with formation of Pro-Pro and tridecapeptide for some unknown reason, it acts on the tridecapeptide with Gly residues at the N- and C-terminals as an endopeptidase, supporting the second possibility. The endolytic action of the enzyme and its inhibition by charged amino and carboxyl groups at the N- and C-terminals would explain why, at a low ratio of enzyme to gelatin, the enzyme produced hexapeptides with apparently susceptible sequences of residues that were resistant to further degradation.

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