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PEPTIDE INHIBITORS OF ANGIOTENSIN I-CONVERTING ENZYME IN DIGESTS OF GELATIN BY BACTERIAL COLLAGENASE

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Summary

Peptide inhibitors of angiotensin I-converting enzyme (peptidyldipeptide hydrolase, EC 3.4.15.1) were produced by digesting gelatin with bacterial collagenase. The inhibitors were isolated from the digests with a combination of alcohol fractionation, treatment with Amberlite CG-50 column, gel filtration through Sephadex G-25, and Dowex 50 column and paper chromatography. Nine peptide fractions were purified to apparent homogeneity judging by thin-layer and ion-exchange column chromatography, and amino acid composition.

Amino acid sequences of the peptides were determined: 2 were found to be mixtures of peptides and the sequence of another was only partially determined.

Six of the peptides were potent inhibitors of the converting enzyme, while the other three were less active. 6 peptides were substrates for the enzyme. The enzyme released a dipeptide, Ala-Hyp from one peptide and was strongly inhibited by this dipeptide. The remainder of the parent peptides was a less effective inhibitor.

Introduction

In 1965, Ferreira [1] found that a low molecular weight fraction from the snake venom (*Bothrops jararaca*) potentiates the activity of bradykinin and named it bradykinin potentiating factor. Amino acid sequences of this factor were determined and named bradykinin potentiating peptides [2,3]. Kato and Suzuki also determined amino acid sequences of three peptides in the Japanese mamushi (*Agkistrodon halys blomhoffii*) venom [4]. Since the amounts of these bradykinin potentiating peptides in both snake venoms were very low,

Abbreviations: ID, inhibition dose.

they were then synthesized by two groups independently [3,4]. It was demonstrated that the potentiating peptides inhibited angiotensin I-converting enzyme or kininase II [5,6].

Except for bradykinin potentiating peptide [2], most other peptide inhibitors in the venoms have a Pro-Pro sequence at their carboxyl terminus [3,4] that resists to cleavage by the converting enzyme [7]. Further study of other small peptide inhibitors may provide information on the mechanism of action of angiotensin I-converting enzyme.

It is well established that clostridiopeptidase A (collagenase, EC 3.4.24.3) has the specificity to hydrolyze preferentially the X-Gly bond in a sequence of Pro-X-Gly-Pro [8]. Thus peptides having a Pro residue at the penultimate position of the carboxyl terminus may be produced in digests of collagen or gelatin by the collagenase.

In this communication, we report production, isolation and determination of the amino acid sequences of peptide inhibitors in the collagenase-digests of gelatin.

Experimental

Materials. Gelatins were obtained from E. Merck AG. Darmstadt, F.R.G. and DIFCO, Detroit, Mich., U.S.A. Trypsin (type I) and α-chymotrypsin (type II) from bovine pancreas, and phenylthiohydantoin-hydroxyproline were purchased from Sigma Chemicals, St. Louis, Mo., U.S.A. An acid carboxypeptidase [9] was a gift from Dr. Yokoyama, Takarasyuzo, Shiga. A collagenase (1000 Mandl units/mg) from *Clostridium histolyticum* was purchased from Seikagaku Kogyo, Tokyo. The converting enzyme from swine kidney cortex was purified by the method described previously [10] and a specific activity of 30 μmol · mg^{-1} · min^{-1} using Benzoyl-Gly-Gly-Gly as substrate.

Isolation of peptide inhibitors from collagenase digests of gelatin. 28 g gelatin were dissolved in 1 l water and 1 mg bacterial collagenase was added. The solution was adjusted to pH 7.2—7.4 by 0.1 M NaOH. After 24 h digestion, the mixture was heated for 15 min in a boiling water bath. The heated solution was concentrated by evaporation and 3 vols. ethanol were added. After standing overnight in the cold, the precipitate was removed by centrifugation at 9000 × *g* for 30 min. The supernatant solution was concentrated and then adjusted to pH 4.5 with conc. HCl and applied to an Amberlite CG-50 column (3 × 45 cm). The column was washed with 1 l 0.01 M sodium acetate buffer (pH 4.5). The eluate was adjusted to neutral pH by 1 M NaOH and concentrated. The concentrate was applied in 3 batches to a Sephadex G-25 column (5 × 80 cm) and eluted with distilled water (10-ml fractions, flow rate 60 ml/h). The active fractions were collected. The fractions were concentrated and adjusted to pH 3.25 with 1 M HCl, before applying to a Dowex 50-X2 column (2.5 × 90 cm) and eluted with 0.2 M sodium acetate buffer (pH 3.25). 3 active fractions, designated F-I, F-II and F-III, were collected and concentrated. After desalting by Sephadex G-25 gel filtration, the peptide fraction was finally isolated by descending paper chromatography using an upper layer of *n*-butanol/acetic acid/water (4 : 1 : 5, v/v) [11].

Purity of peptide inhibitors. The purity of the peptide inhibitors was

checked by three differen methods: (1) Avicel thin-layer chromatography using 8 kinds of solven mixture; (2) cation-exchange column chromatography using a JEOL amino acid analyzer 5 AH; and (3) amino acid analyses.

Chemical analysis. Amino acid analyses of each peptide inhibitor were done after hydrolysis by 6 M HCl for 24 h at 110°C. Determination of molecular weight of peptide was done by two methods; (1) minimum molecular weight was calculated from amino acid composition of the peptide, and (2) maximum molecular weight was determined by *N*-terminal analysis of peptide using the analyzer and Gly-Gly as standard. Determination of amino acid sequence of peptide inhibitors was mainly carried out by the direct method of Edman [12,13]. The carboxyl terminal amino acid sequence of some peptides was determined by treatment with acid carboxypeptidase. The peptide (0.5 μmol) was digested by 132 μg enzyme in 7.5 ml 0.1 M sodium acetate buffer (pH 5.2) at 37°C. A 0.75-ml aliquot at 0, 0.25, 0.5, 1, 2, 4, 8 and 24 h was added to 0.75 ml 10% trichloroacetic acid. Amino acids released were determined by the analyzer.

Assay of the converting enzyme and inhibitors. The activity of converting enzyme was measured by the method described previously [14]. Inhibitory activity of peptide was also determined as described previously [14]. The ID_{50}, defined as the concentration which inhibits 50% of the enzyme activity, was determined using several concentrations of a peptide inhibitor. Total ID was defined as the quotient of the weight of materials divided by its ID_{50}.

Results

Though inhibition of the activity of converting enzyme by low molecular weight peptides has been reported [15—18], there has been no report of inhibition of the enzyme by proteins, except for antibody [19,20]. The effect of gelatin from two different sources on the enzyme was examined. The activity of the enzyme was inhibited depending on the amount of gelatin used. The enzyme activity was inhibited to a greater extent by gelatin from Merck than from DIFCO at the same amount. Since commercial gelatins are usually impure, it is possible that some contaminating materials contributed to non-specific inhibition of the enzyme. However, gelatin appeared to have no effect on carboxypeptidase A, another metalloenzyme [21]. Assuming 100 000 as the molecular weight of gelatin from Merck [22], the ID_{50} of the protein was calculated to be $7.1 \cdot 10^{-5}$ M.

When inhibitory activities of the two gelatins digested by the bacterial collagenase for 2.5 h were measured, they were markedly increased by treatment with the collagenase in comparison with the intact proteins. The enzyme activity was inhibited by the digest from Merck more than twice as much as it was by that from DIFCO. Thus, the following experiments were done using gelatin from Merck as starting materials.

Time dependence of inhibitor formation was examined with digesting gelatin by the collagenase for 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 6 and 24 h. The formation was time-dependent and maximum formation was obtained after 24 h digestion.

Isolation of peptide inhibitors from 24-h digests was attempted by the proce-

dure described in Experimental. The procedure was based on a combination of alcohol fractionation, Amberlite CG-50 column chromatography, Sephadex G-25 gel filtration, and Dowex 50 column and paper chromatography. The non-adsorbed fraction from an Amberlite CG-50 column was separated by Sephadex G-25 (Fig. 1). Potent inhibitors mainly appeared in tubes 110—124, corresponding roughly to hexa- and nonapeptides. The fractions of large peptide eluted near the void volume did not show any inhibitory activity. The collagenase predominantly produced two tripeptides, Gly-Pro-Hyp and Gly-Pro-Ala [23], but these peptides would appear in tubes 120—140, in which there was little inhibitory activity for the enzyme. When the active fractions were further fractionated with the Dowex 50 column, four inhibitor peaks were obtained under the conditions used. Since the first peak had less inhibitory activity based on the amount of peptide in this fraction, it was discarded. The other active peaks designated as F-I, F-II and F-III, in the order of elution were further separated by preparative paper chromatography. 3, 9 and 8 peptide bands were obtained by this procedure from F-I, F-II and F-III, respectively. The purification procedure is summarized in Table I. Recovery of total ID from Amberlite CG-50 was lower than that of the next step. Since the activity of converting enzyme was inhibited by acetate ion [14], the experimental control included this ion. Because the peptides would be expected to compete with acetate, the activity of the enzyme might be overestimated in this fraction. The sums of recoveries of total ID in F-I, F-II and F-III, and of preparative paper chromatography were low. Compared with the ID_{50} of a fraction obtained through Sephadex G-25, those of F-I, F-II and F-III, and of the paper chromatography were almost the same or slightly higher. This fact suggests that the

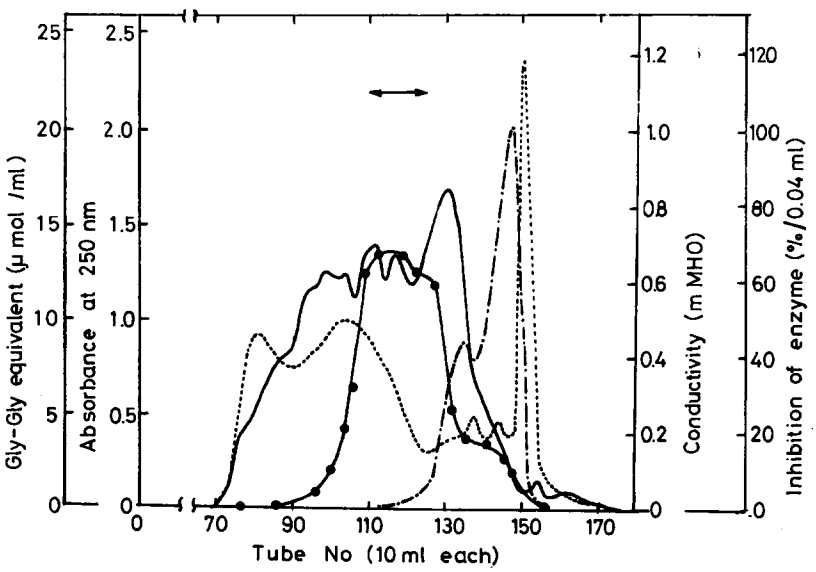

Fig. 1. Purification of the inhibitors through Sephadex G-25 gel filtration. Non-adsrobed fraction (6.13 g/12 ml) from Amberlite CG-50 was applied to a Sephadex G-25 column (5 × 80 cm) and eluted with water. Absorbance at 250 nm (· · · · ·), conductivity (— · — · —), content of amino groups (———) and inhibitory activity (●———●) were measured on each fraction.

TABLE I

SUMMARY OF PURIFICATION OF INHIBITORS FROM DIGESTS OF GELATIN BY BACTERIAL COLLAGENASE

Step		Amount (mg)	ID_{50} (μg)	Total ID	Recovery (%)
Gelatin		28 000	706	3 966	
1. Digests		28 000	147	190 000	100
2. 75% Ethanol supernatant		18 000	82	225 488	118.4
3. Amberlite CG-50		12 380	103	120 194	63.1
4. Sephadex G-25		2 578	15	171 867	90.2
5. Dowex 50 X 2					
F-I		168	18	9 333	4.9
F-II		310	26	11 923	6.3
F-III		254	20	12 700	6.7
6. Paper chromatography					
F-I	Rf 1	33	49	674	0.35
	Rf 2	32.5	27	1 204	0.63
	Rf 3	51	40	1 275	0.67
F-II	Rf 2	4.9	8.4	583	0.31
	Rf 3	7.3	6.1	1 197	0.63
	Rf 4	19.4	42	462	0.24
	Rf 5	3.6	4.0	900	0.47
	Rf 6	1.7	4.4	386	0.20
	Rf 7	10.5	145.5	72	0.04
	Rf 8	11.6	11	1 055	0.55
	Rf 9	24.3	27.5	1 617	0.85
F-III	Rf 3	36.4	— *		
	Rf 4	39.9	72	554	0.28
	Rf 5	11.5	17.3	665	0.34
	Rf 7	16.1	64.9	248	0.13

* Not determined due to low activity.

fraction from Sephadex G-25 contained almost peptide inhibitors only, and the following procedures constitute isolation but not purification of each peptide from the mixtures. After elution of F-I, F-II and F-III, the Dowex 50 column was washed with 0.01 M NaOH containing 0.2 M NaCl to elute two additional peptides (1.12 g). However, inhibitory activities of these fractions were less than those of F-I, F-II or F-III. Although potentiation of an inhibitor by mixing with others was examined, only an additional effect was observed in every case.

Some impure peptide fractions (all of 3 fractions of F-I, R_F 4 and 8 of F-II, and R_F 4 of F-III) were omitted from further study by checking their homogeneity with Avical thin-layer chromatography. All of the remaining 9 peptide fractions showed a single peak under cation-exchange column chromatography using the analyzer. All of the peptide fractions gave reasonable ratios of each amino acid (Table II). Based on the 3 different methods described above, 9 peptide fractions were judged as homogeneous.

The molecular weight of each peptide was deduced by 2 independent methods. Except for R_F 3 of F-II and R_F 7 of F-III, both of minimum and maximum quantities for the peptides were roughly equal (Table II). Questionable values obtained for R_F 3 of F-II and R_F 7 of F-III were explained as being the result of the fact that these fractions were mixtures of approximately equal amounts of two peptides, as shown by sequential studies described below.

TABLE II

AMINO ACID COMPOSITIONS AND MOLECULAR WEIGHT OF INHIBITORS

Inhibitor (R_F)		Amino acid recovered (μmol/mg)	Molecular weight	
			Minimum *	Maximum **
F-II	2	Hyp(2.65), Asp(1.23), Thr(1.11), Pro(1.63) Gly(3.72), Ala(1.06)	800	667
	3	Hyp(3.64), Pro(3.01), Gly(3.73), Ala(1.18)	794	440
	5	Hyp(2.00), Pro(2.33), Gly(4.41), Ala(4.17)	485	478
	6	Hyp(2.08), Pro(4.15), Gly(3.84), Ala(2.00)	511	424
	7	Hyp(1.18), Ser(0.87), Pro(2.90), Gly(4.24), Ala(2.08), Ile(1.05)	993	998
	9	Hyp(1.28), Ser(1.06), Pro(1.50), Gly(4.03), Ala(1.44), Val(1.23), Ile(1.27)	780	675
F-III	3	Hyp(3.63), Pro(3.88), Gly(3.72)	303	281
	5	Pro(3.02), Gly(4.64), Ala(6.20)	468	436
	7	Hyp(1.08), Pro(3.67), Gly(3.83), Ala(1.23), Val(1.11), Ile(0.92)	1031	436

* Calculated from amino acid composition.

** Determined from N-terminal analysis of peptide using the analyzer and Gly-Gly as a standard peptide.

The amino acid sequence of the pure peptides was mainly determined by the direct method of Edman. The sequence of the carboxyl terminal region of some of the peptides was determined by treatment with an acid carboxypeptidase. The results obtained are summarized in Table III. The complete structure of R_F 7 of F-II could not be determined because of a loss of peptide during the

TABLE III

AMINO ACID SEQUENCE OF INHIBITORS AND THEIR ID_{50} VALUES

Inhibitor (R_F)		Amino acid sequence	ID_{50} (μM)
F-II	2	Gly-Pro-Hyp-Gly-Thr-Asp-Gly-Ala-Hyp	10.5
	3	Gly-Pro-Hyp-Gly-(Ala/Pro)-Hyp *	11.3 ***
	5	Gly-Pro-Ala-Gly-Ala-Hyp	8.3
	6	Gly-Pro-Pro-Gly-Ala-Hyp	8.6
	7	Gly-Pro-Hyp-Gly-Ala-Ile-Gly-Pro- (Gly, Ser, Pro, Ala)	146.5
	9	Gly-Pro-Ile-Gly-Ser-Val-Gly-Ala-Hyp	31.8
F-III	3	Gly-Pro-Hyp	n.d.
	5	Gly-Pro-Ala-Gly-Ala-Pro-Gly-Ala-Ala	37.0
	7	Gly-Pro-(Ile/Val)-Gly-Pro-(Hyp/Ala) **	123.4 ***
Commercials		Gly-ProHyp	250
		Gly-Pro-Ala	405

* Ratio of Ala : Pro = 44.4 : 55.6.

** Ratio of Ile : Val = 45.3 : 54.7 and Hyp : Ala = 46.8 : 53.2.

*** Values based on average molecular weight.

n.d., not determined.

→ Edman method, ← acid carboxypeptidase.

procedure. R_F 3 of F-II was an almost equal mixture of two hexapeptides, Gly-Pro-Hyp-Gly-Pro-Hyp and Gly-Pro-Hyp-Gly-Ala-Hyp. R_F 7 of F-III was also a mixture of peptides. Four amino acid sequences for this fraction were theoretically possible from 2 substitutions. The molecular weight determined for this fraction indicated, at most, a mixture of two peptides. Comparing ratios of Ile/Val and Hyp/Ala, probable amino acid sequences of this fraction may be Gly-Pro-Ile-Gly-Pro-Hyp and Gly-Pro-Val-Gly-Pro-Ala.

Of all the peptides determined their primary structures were tri-, hexa-, nona- and dodeca-peptides and had the Gly-Pro sequence at the *N*-terminus, consistent with the specificity of the collagenase. It was obvious that the commercial collagenase preparation did not contain any other proteinases or peptidases interfering with the enzyme.

Many of the peptides had the Ala-Hyp sequence at their carboxyl terminus and had potent inhibitory activity. However some inhibitors (R_F 3 and 7 of F-III) having C-terminal Pro-Hyp showed weak activities for the converting enzyme. The results clearly indicate that the converting enzyme was inhibited more potently by peptide having a structure of Ala-Hyp than Pro-X at their C-terminus.

It was expected that peptide inhibitors having Ala in the penultimate position of the C-terminus would be cleaved by the converting enzyme. Since amino acids and homo-oligopeptides could be separated with the molecular sieving effect of a cation-exchange resin [24], this method was adapted to confirm susceptibility of the inhibitors to the enzyme. An example of the result obtained on R_F 9 of F-II is shown in Fig. 2. The digest of the peptide by the enzyme emerged later that the intact peptide did, demonstrating the hydrolysis of the peptide by the enzyme. Susceptibilities of other peptide inhibitors are

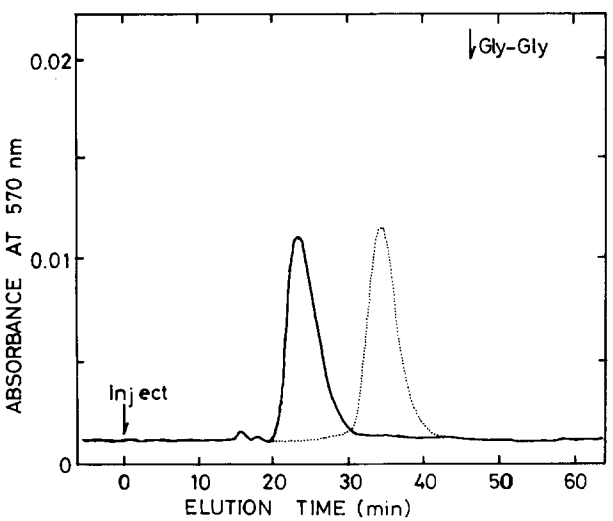

Fig. 2. Cation-exchange column chromatography of digests from R_F 9 of F-II by the converting enzyme. Peptide (50 μg) was digested by 0.5 μg enzyme in 0.1 ml 0.1 M Tris-HCl (pH 8.0) with 0.1 M NaCl for 5.5 h at 37°C. After addition of 0.9 ml 0.2 M citric acid, the sample was chromatographed using a short column amino acid analyzer and 0.2 M sodium citrate buffer (pH 3.25). (———), intact; (· · · · ·), digests.

summarized in Table IV. Because the converting enzyme could not cleave the imino bond of the peptide [25], the results obtained reflected this specificity of the enzyme. R_F 3 of F-II was expected to be a mixture of hydrolyzable and resistant peptides based on the sequential study. After digestion of this fraction by the enzyme, the digest gave a lower corresponding peak to that from the intact peptide in addition to a new peak. This fact supports contention that R_F 3 of F-II is a mixture of 2 peptides having the structures presented in Table III. Since R_F 7 of F-II could not be hydrolyzed by the enzyme, the peptide might have a Pro residue at the penultimate position of the C-terminus. All of the susceptible peptides kept the almost same inhibitory effect on the enzyme after extensive hydrolysis of the peptides by the enzyme (Table IV). The inhibition of the resistant peptides should be unchanged before and after the treatment. Since relatively large amount of the resistant peptides was used to detect their activities, trace impure in these fractions might be a reason of variations observed.

Then R_F 9 of F-II was extensively hydrolyzed by the converting enzyme. The digests were separated with descending paper chromatography using a solvent [11]. Inhibitory activity and amino acid composition of 3 peptide fragments obtained were determined. Two peptide fragments derived from the N-terminal part of the parent inhibitor had low inhibitory effect on the enzyme. A dipeptide, Ala-Hyp, had the strongest inhibitory effect on the enzyme among 3 fragments, indicating that the dipeptide, a product released by the enzyme, was an inhibitory substance. Though another dipeptide, Val-Gly, should have been produced from the modified heptapeptide, it may have been lost in the chromatography by solvent overrun.

The question was whether bacterial collagenase, only being an exogenous enzyme of the mammalian species, produced specific inhibitors for the mammalian converting enzyme from gelatin. Thus gelatin was digested by

TABLE IV

SUSCEPTIBILITY TO THE CONVERTING ENZYME AND INHIBITORY ACTIVITY OF PEPTIDE INHIBITORS

An adequate amount of peptide depending on its inhibitory activity was preincubated overnight at 37°C by 0.5 μg the converting enzyme. After heating for 5 min, inhibitory activity of the whole solution was determined by the addition of the enzyme and substrate. The same amount of peptide kept in the cold without enzyme served as control.

Inhibitor (R_F)		Susceptibility *	Inhibition of enzyme (%)	
			Control	Preincubated
F-II	2	+	29	32
	3	+	31	30
	5	+	19	15
	6	+	35	26
	7	—	66	86
	9	+	44	45
F-III	3	—	59	47
	5	+	43	40
	7	—	49	71

* Determined by the method described in legend of Fig. 2.

mammalian proteinases, trypsin and α-chymotrypsin. Both endogenous digestive enzymes produced peptide inhibitors in the digests of gelatin. Trypsin formed peptides having more potent inhibitory activity than α-chymotrypsin did. The facts indicate that production of peptide inhibitors in digests of gelatin is not limited to the bacterial collagenase.

Discussion

We made an attempt to produce peptide inhibitors for the converting enzyme from a protein by porteinase digestion. Since gelatin is rich in proline [26] forming imino bonds in peptides which resisted to cleavage by usual proteinases and could be cheaply obtained from various commerical sources, it was used as starting material. The bacterial collagenase was selected as an enzyme for digestion of gelatin based on its specificity [8]. We found that the collagenase produced peptide inhibitors from gelatin for the converting enzyme. The peptide inhibitors were purified from gelatin-digests to confirm their amino acid sequences and their inhibitory protencies for the converting enzyme. 9 apparently pure peptide fractions were obtained with five successive procedures. The primary structure of these homogeneous peptides was determined by combination of the direct method of Edman and treatment with an acid carboxypeptidase.

The inhibitory potency of peptides having a C-terminal Ala-Hyp structure was the same order as those of SQ 20718 and 20859 found in Brazilian snake venom and determined using a synthetic substrate [7]. However, the peptide having C-terminal Pro-X sequences from the gelatin-digests were less effective than any kinds of bradykinin potentiating peptide having C-terminal Pro-Pro sequences. Susceptible peptides had roughly similar inhibitory potency on the enzyme in each other and kept completely their activities after pre-treatment by the converting enzyme. Moreover N-terminal fragments from R_F 9 of F-II had less inhibitory potencies than a C-terminal dipeptide, Ala-Hyp, did. These findings indicate that N-terminal parts of susceptible inhibitor have little contribution for inhibition of the enzyme. On the other hand, it is suggested that N-terminal region of the resistant peptides has an important role to decide their inhibitory activities.

Though the converting enzyme has relatively broad substrate specificity as peptidyldipeptide hydrolase [15,25], the biologically active, natural substrates are bradykinin, a nonapeptide, and angiotensin I, a decapeptide. The bradykinin potentiating peptides found in both Brazilian and Japanese snake venoms were penta- to triceda-peptides. The inhibitors isolated from the gelatin-digests were also tri- to dodeca-peptides. Furthermore large peptides eluted near the void volume by Sephadex G25 gel filtration as shown in Fig. 1 had no inhibitory effect on the converting enzyme activity. These facts suggest that the molecular size of a peptide is important with respect to the potency of its inhibitory effect on the enzyme in addition to its primary structure.

Since the ID_{50} of the peptides having an Ala-Hyp at their C-terminus was approx. 1/200 of the pentapeptide, Bradykinin potentiating peptide 5a, a relatively large amount of inhibitor was used to determine the activity for the enzyme. If the pentapeptide were used at a high concentration to detect

dipeptide activity, Ala-Pro, the peptide might still have its inhibitory effect on the enzyme after pre-incubation.

Peptide inhibitors were produced from gelatin not only by the bacterial collagenase digestion but also by mammalian digestive enzymes, trypsin and α-chymotrypsin digestions. Gelatin is a food with low nutrient value. This is the first observation that biologically active peptides are produced from food proteins by digestive proteinases.

Finally infections by species of *Clostridia* capable of producing collagenase sometimes cause shock in the victim. Since this phenomenon can not be caused by the toxin of the bacteria alone [27], it has been suggested that the syndrome may be produced by secondary metabolites formed during the infection [28]. Because the bacterial collagenase digests gelatin and releases peptide inhibitors of the converting enzyme, the shock may partially be caused by inhibition of the converting enzyme activity by peptide inhibitors produced from tissue collagen by bacterial collagenase.

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