Formations of Oligopeptides from Dipeptides by the Protease from *Streptomyces cellulosa*

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The protease from *Streptomyces cellulosa* formed more turbidity in a 16% soybean protein hydrolysate in the initial stage of the reaction than α -chymotrypsin did, when the proteolytic activity of the protease was same as that of α -chymotrypsin. In highly concentrated solutions (2.5%) of various dipeptides, oligopeptides were produced by condensation by the protease. The oligopeptides formed were (L-Leu-Gly)₂ and (L-Leu-Gly)₃ from L-Leu-Gly, (L-Phe-L-Val)₂ from L-Phe-L-Val, (L-Val-L-Phe)₂ and (L-Val-L-Phe)₃ from L-Val-L-Phe, and (L-Leu-L-Met)₂ and (L-Leu-L-Met)₃ from L-Leu-L-Met.

The reverse reaction of proteases, *i.e.*, peptide bond synthesis, can also be catalyzed by the same enzyme, as was first demonstrated by Bergmann and Fraenkel-Conrat¹⁾ in 1937 with papain which catalyzed amide (anilide) bond synthesis. Since then, many reports have been appeared on reverse reactions of proteases, for instance, semi-synthesis of human insulin from porcine insulin,^{2~4)} semi-synthesis of an angiotensin analogue,⁵⁾ or plastein formation.^{6~8)} However, proteases used for peptide synthesis are enzymes obtained mainly from animals and plants, and the equilibrium of the reaction lies toward hydrolysis. We are interested in screening microorganisms which secrete proteases having strong activity in peptide synthesis.

The protease from *Streptomyces cellulosa* formed more turbidity in a 16% soybean protein hydrolysate in the initial stage of the reaction than α -chymotrypsin did, so this protease presumably catalyzes the condensation or the transpeptidation reaction of peptides in highly concentrated peptide solutions. This study deals with the formations of oligopeptides by the protease from *S. cellulosa*, using high concentrations of various dipeptide solutions as the substrates.

MATERIALS AND METHODS

Materials. Gly, L-Phe, and L-Leu-Gly were purchased from the Nakarai Chemical Company. All the other peptides were purchased from the Sigma Chemical Company. The protease from *Streptomyces cellulosa* was purified to homogeneity on electrophoretic analysis as described in our previous report.⁹⁾

Assay of the turbidity-forming activity. Turbidity formation by the protease was measured according to the method described in our previous report.⁹⁾

Estimation of molecular weight of oligopeptides by gel filtration. A column of Bio-Gel P-2 (400 mesh) washed with 0.01 M phosphate buffer (pH 7.0) containing 0.1 M NaCl, was used. Gly and standard peptides (carbobenzoyl-L-Pro-L-Leu-Gly, hexa-Gly, penta-Gly, tetra-Gly, tri-Gly, and di-Gly) were applied on the Bio-Gel P-2 column (1.7 × 75 cm). Blue dextran was used to determine the void volume of the column. Fractions of 1.5 ml were collected at flow rate of 8.4 ml/hr.

Analytical methods.

(i) **N-Terminal amino acid analysis.** The N-terminal amino acids of peptides were analyzed by the method of Sanger and Thompson.¹⁰⁾ The dinitrophenyl peptides were hydrolyzed in 6 N HCl in an evacuated tube for 16 hr at 105°C. The dinitrophenyl-amino acids (DNP-amino acids) were identified by two-dimensional thin-layer chromatography (TLC) in the solvent systems of toluene-pyridine-2-chloroethanol (30:9:18) treated with 18 ml of 0.8 N ammonia water, and benzene-pyridine-acetic acid (80:20:2).¹¹⁾ In quantitative analysis of DNP-amino acids, the silica gel powder at the DNP-amino acid

position was scratched off of the TLC plate and extracted in 4 ml of 1% NaHCO_3 . Then the optical density of the extract was measured at 360 nm with a Shimadzu UV-210A spectrophotometer.

(ii) *C-terminal amino acid analysis.* The C-terminal amino acids of peptides were analyzed by the method of Akabori.¹²⁾ The dried sample was dissolved in 0.5 ml of anhydrous hydrazine, and was hydrolyzed in an evacuated tube for 8 hr at 100°C. After opening the tube, the sample was lyophilized and then analyzed using a Hitachi KLA-5 automatic amino acid analyzer.

(iii) *Amino acid analysis.* Hydrolysis of peptides was carried out in 6N HCl in an evacuated tube for 20 hr at 110°C. Only products from L-Leu-L-Met were oxidized by performic acid and then hydrolyzed. The amino acids produced were analyzed with a Hitachi KLA-5 automatic amino acid analyzer.

Estimation of retention constants of oligopeptides by reverse phase high performance liquid chromatography. Retention constants of oligopeptides were determined by the method of Molnár and Horváth.¹⁴⁾ A 5- μm LiChrosorb RP-18 column (0.5 $\times$ 25 cm) was used. The flow-rate and column temperature were 2 ml/min and 70°C, respectively. The mobile phase was 0.5 M perchloric acid and the mobile phase modifier was acetonitrile. The concentration of the mobile phase modifier was increased linearly from 0 to 100% (v/v) over 60 min. The elution pattern was monitored by absorption at 220 nm. L-Phe and standard peptides (di-Phe, tri-Phe, tetra-Phe, and penta-Phe) were applied on the above column. In Fig. 1, the retention times observed were plotted against $\ln(1 + \sum_j D_j n_{ij})$, where D_j is the calculated retention con-

FIG. 1. Relationship between Retention Time and $\ln(1 + \sum_j D_j n_{ij})$.

The observed retention times of samples were plotted against $\ln(1 + \sum_j D_j n_{ij})$, where D_j was the calculated retention constant.¹³⁾ 1, phenylalanine; 2, di-phenylalanine; 3, tri-phenylalanine; 4, tetra-phenylalanine; 5, penta-phenylalanine.

stant of amino acid j by unweighted curve fitting, and n_{ij} is the number of residues of amino acid j in peptide i .¹⁵⁾

Paper chromatography. Paper chromatography was carried out on Tōyō No. 50 filter paper (40 $\times$ 40 cm) using n -butanol, acetic acid and water (4:1:2, v/v) as a solvent system by the ascending method. The amino acids and peptides on the paper chromatogram were detected with ninhydrin reagent.

Digestions of P_1 and P_2 by carboxypeptidase A (CPase A). In order to determine the C-terminal amino acids of the products (P_1 and P_2) from L-Leu-Gly, they were hydrolyzed by CPase A at pH 7.5.¹³⁾ After the appropriate time of incubation at 37°C, the reaction mixture was subjected to paper chromatography. Each spot stained by ninhydrin reagent was cut out and the content of each fraction was determined by the optical density at 570 nm with a Shimadzu UV-210A spectrophotometer.

RESULTS

Actions of the protease on high concentrations of various dipeptides

Each 5% dipeptide solution (100 μl) adjusted to pH 7.0 was incubated with the protease (100 μl , turbidity-forming activity, 26.4 U). After 0, 3, and 8 hr incubations at 40°C, the reaction products were analyzed by paper chromatography. One or two unknown spots which were stained with ninhydrin were observed in each case. These results are summarized in Table I. On the other hand, strong turbidity formation could be observed using L-Phe-L-Val, L-Val-L-Phe, and L-Leu-L-Met as substrates. However, such phenomena were not observed even in 8 hr incubation of the other dipeptides.

Action of the protease on L-Leu-Gly

The paper chromatogram of products from L-Leu-Gly by the protease is shown in Fig. 2. Two unknown spots (P_1 and P_2) which were stained by ninhydrin were observed, along with the hydrolysis products (Leu and Gly). The areas around each stained region (P_1 and P_2) were cut out, eluted with water and then lyophilized. The preparations of P_1 and P_2 were homogeneous on paper chromatography and used in following analytical experiments. The yields of P_1 and P_2 from 500 mg of L-Leu-Gly after 8 hr of incubation were 1.2 mg and

TABLE I. ACTIONS OF THE PROTEASE ON HIGH CONCENTRATIONS OF VARIOUS DIPEPTIDES

Dipeptides	No. of unknown spots ^a	Turbidity ^b
L-Phe-L-Leu-NH ₂	0	—
Gly-L-Leu	2	—
Gly-L-Ile	1	—
Gly-L-Phe	2	—
L-Ala-L-Leu	1	—
L-Leu-Gly	2	—
L-Leu-L-Ala	1	—
L-Leu-L-Ser	2	—
L-Leu-L-Val	1	—
L-Phe-L-Val	1	+
L-Val-L-Phe	1	+
L-Leu-L-Met	1	+

^a No. of unknown spots means the numbers of spots detected from paper chromatography, aside from hydrolytic products (amino acids) and dipeptides as substrates.

^b This column shows that the phenomenon of turbidity formation was observed or not within 1 hr of incubation by the protease from *S. cellulosa*.

22.5 mg, respectively.

Estimation of molecular weights of P_1 and P_2

The molecular weights of P_1 and P_2 were estimated to be 390 and 510 from the elution patterns by gel filtration on a Bio-Gel P-2 column (Fig. 3).

Amino acid compositions and N-terminal and C-terminal amino acids of P_1 and P_2

By amino acid analysis, the molar ratios of Leu and Gly were 1:1.1 in P_1 and 1:1.0 in P_2 , respectively, as shown in Table II. The N-terminal and C-terminal amino acids of P_1 and P_2 were identified to be Leu and Gly, respectively. The amino acid composition of the residues in which the DNP-amino acid was removed by ethyl ether extraction was analyzed (Table II). The ratio of amino acid composition of the residue prepared from P_1 was Leu and Gly (1:1.9). By considering the content of DNP-Leu obtained from P_1 in N-terminal amino acid analysis, the ratio of DNP-amino acid and amino acid composition of the residue was DNP-Leu, Leu and Gly

FIG. 2. Paper Chromatogram of Products from 2.5% L-Leu-Gly by the Protease.

E, the protease from *S. cellulosa*; G, glycine; L, leucine; C, 2.5% L-Leu-Gly incubated without enzyme for 8 hr at 40°C; P_1 , P_2 , unknown spots stained with ninhydrin reagent.

FIG. 3. Molecular Weights of Oligopeptides by Gel Filtration on a Bio-Gel P-2 Column.

○, P_1 ; △, P_2 . 1, carbobenzoxy-L-Pro-L-Leu-Gly; 2, hexa-glycine; 3, penta-glycine; 4, tetra-glycine; 5, tri-glycine; 6, di-glycine; 7, glycine; V_e , elution volume; V_o , void volume of the column.

(1.0:1:1.9). With the same method, the ratio obtained from P_2 was DNP-Leu, Leu and Gly (0.9:2:3.2).

Hydrolyses of P_1 and P_2 by CPase A

Figure 4(a) shows that almost identical con-

TABLE II. AMINO ACID COMPOSITIONS AND N- AND C-TERMINAL AMINO ACIDS OF P₁ AND P₂ PRODUCED FROM L-Leu-Gly BY THE PROTEASE

Product	Molar ratio of amino acid	Treatment with FDNB ^a			Hydrolysis by hydrazine
		Molar ratio of amino acids			
	Leu : Gly	DNP-Leu : Leu : Gly			
P ₁	1 : 1.1	1.0	: 1	: 1.9	Gly
P ₂	1 : 1.0	0.9	: 2	: 3.2	Gly

^a FDNB, 1-fluoro-2,4-dinitrobenzene. FIG. 4. Hydrolyses of Products P₁ and P₂ by Carboxypeptidase A.(a) P₁ was digested by CPase A for 1, 3, and 6 hr at 40°C.(b) P₂ was digested by CPase A for 0.5, 1, and 3 hr at 40°C. ○—○, leucine; ●—●, glycine; △—△, L-Leu-Gly; □—□, P₁.

tents of Leu, Gly, and Leu-Gly were simultaneously released from P₁ by CPase A digestion. Figure 4(b) also shows that almost identical contents of Leu, Gly, and P₁ were simultaneously released from P₂ by CPase A digestion in the initial stage of the reaction, followed by the production of Leu-Gly.

Actions of the protease on L-Phe-L-Val, L-Val-L-Phe and L-Leu-L-Met

1 ml of the protease solution (turbidity-forming activity, 264 U) was added to 1 ml of 5% dipeptide solution (L-Phe-L-Val, L-Val-L-Phe or L-Leu-L-Met) adjusted to pH 7.0 at 40°C. After 1 hr of incubation, strong turbidity could be observed in each dipeptide solution. The reaction mixture (20 μ l) was subjected to paper chromatography (Fig. 5). The unknown spots (P_p, P_v and P_L) were observed from L-Phe-L-Val, L-Val-L-Phe and L-Leu-L-Met on

paper chromatograms, respectively, along with the spots of free amino acids produced by hydrolysis of the dipeptides.

Then P_p, P_v and P_L were separated from the reaction mixtures by centrifugation, washed sufficiently with ice-water, and dried to constant weight over P₂O₅ under reduced pressure. The P_p, P_v and P_L obtained were homogeneous on paper chromatography, and their R_f values were the same as those shown in Fig. 5. These homogeneous samples of P_p, P_v and P_L were used in the following experiments. The yields of P_p, P_v and P_L from 50 mg of dipeptide were 15.3, 8.3 and 8 mg, respectively, with 3 hr of incubation.

Amino acid compositions and N-terminal and C-terminal amino acids of P_p, P_v and P_L

The molar ratios of amino acid compositions of P_p, P_v and P_L were Phe and Val (1 : 1)

FIG. 5. Paper Chromatograms of Products P_p , P_v , and P_L Produced from 2.5% Dipeptides by the Protease. (a) With L-Phe-L-Val (PV) as a substrate: E, the protease from *S. cellulosa*; P, phenylalanine; V, valine; P_p , unknown spot stained with ninhydrin reagent.

(b) With L-Val-L-Phe (VP) as a substrate: P_v , unknown spot stained with ninhydrin reagent.

(c) With L-Leu-L-Met (LM) as a substrate. L, leucine; M, methionine; P_L , unknown spot stained with ninhydrin reagent.

C, 2.5% of each dipeptide incubated without enzyme for 8 hr at 40°C.

TABLE III. AMINO ACID COMPOSITIONS AND N- AND C-TERMINAL AMINO ACIDS OF P_p , P_v , AND P_L PRODUCED FROM VARIOUS DIPEPTIDES BY THE PROTEASE

Product	Molar ratio of amino acid	Treatment with FDNB ^a	Hydrolysis by hydrazine
P_p	Phe: Val 1.04: 1	Phe	Val
P_v	0.99: 1	Val	Phe
P_L	Leu: Met 1.01: 1	Leu	Met

^a FDNB: 1-fluoro-2,4-dinitrobenzene.

in both P_p and P_v , and Leu and Met (1:1) in P_L . The N-terminal amino acids of P_p , P_v and P_L were Phe, Val and Leu, respectively. On the other hand, the C-terminal amino acids of P_p , P_v , and P_L were Val, Phe and Met, respectively. These data are summarized in Table III.

Retention constants of P_p , P_v and P_L

P_p , P_v and P_L were dissolved in 0.5 M perchloric acid and applied on a LiChrosorb RP-

TABLE IV. RETENTION CONSTANTS OF P_p , P_v AND P_L

Samples		Retention constant ^a $\sum D_j n_{ij}$
Substrate	Product	
L-Phe-L-Val		38.8
	P_p	90.0
L-Val-L-Phe		38.8
	P_{v1}	89.9
	P_{v2}	111.2
L-Leu-L-Met		40.9
	P_{L1}	82.5
	P_{L2}	111.1

^a These values were calculated from the calibration curve in Fig. 1.

18 column. The retention constant ($\sum D_j n_{ij}$) of P_p was calculated to be 90.0 from the calibration curve in Fig. 1, as its retention time was observed to be 24.33 min. This value of the retention constant was about twice that (38.8) of L-Phe-L-Val. On the other hand, two retention times (24.43 and 27.5 min) were observ-

ed from P_v . Therefore, retention constants of P_v were calculated to be 89.9 and 111.2. These values were about twice or three times that (38.8) of L-Val-L-Phe. In the case of P_L , two retention times (23.07 and 27.54 min) were also observed. Therefore, retention constants were calculated to be 82.5 and 111.1. These values were also twice or three times that (40.9) of L-Leu-L-Met. These data are summarized in Table IV.

DISCUSSION

In our previous report,⁹⁾ we showed that the protease from *S. cellulosa* forms much turbidity from soybean protein hydrolysate in the initial stage of the reaction. The present study investigated the actions of the protease on various dipeptides at high concentrations. As a result, it was recognized that unknown products stained by ninhydrin were produced from high concentrations of various dipeptide solutions (Table I).

The structures of the products (P_1 , P_2 , P_p , P_v and P_L) were analyzed by various methods. P_1 was identified as Leu-Gly-Leu-Gly from the results of amino acid composition, N-terminal amino acid, C-terminal amino acid, molecular weight, and CPase A digestion of P_1 .

By the same methods, it can be presumed that the structure of P_2 is Leu-(Leu, Leu, Gly, Gly)-Gly. Moreover, the result of CPase A digestion supports the structure (Leu-Gly-Leu-Gly-Leu-Gly) for P_2 , because the same contents of Gly, Leu, and P_1 were simultaneously liberated from P_2 in the initial stage of the reaction and the liberation of Leu-Gly was also observed later.

From the results of amino acid composition, N-terminal amino acid, C-terminal amino acid, and retention constant of P_p , two possible structures of P_p were presumed to be Phe-Val-Phe-Val and Phe-Phe-Val-Val. The structure Phe-Val-Phe-Val for P_p was supported by the results of the structural analyses of P_1 and P_2 .

P_v is presumed to be a mixture of Val-Phe-Val-Phe (P_{v1}) and Val-Phe-Val-Phe-Val-Phe

(P_{v2}), from the results of amino acid composition, N-terminal amino acid, C-terminal amino acid and retention constants of P_v .

By the same methods as those for P_v , it can be presumed that P_L is a mixture of Leu-Met-Leu-Met (P_{L1}) and Leu-Met-Leu-Met-Leu-Met (P_{L2}) from the results of amino acid composition, N-terminal amino acid, C-terminal amino acid, and retention constants of P_L . These data are summarized in Table III and Table IV.

These results show that the protease from *S. cellulosa* catalyzes the condensation reaction to produce tetrapeptides and hexapeptides at high concentrations of dipeptides. However, α -chymotrypsin did not produce oligopeptides (tetrapeptides or hexapeptides) under the same conditions. Furthermore, there are many reports in which the various proteases^{5,16~18)} such as α -chymotrypsin, papain, thermolysin, etc., catalyze the condensation reactions from N-protected amino acids (or peptides) and C-protected amino acids (or peptides). However, there is no report relating to a condensation reaction of N- and C-unprotected dipeptides by proteases. The present study shows that the protease from *S. cellulosa* is a novel enzyme which catalyzes a condensation reaction of N- and C-unprotected dipeptides.

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