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Synthesis of Peptides by Bovine Gastricsin

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Abstract: Bovine gastricsin catalyzes peptide synthesis over an optimum range of pH 4~5, resulting in satisfactory yields of methyl esters and *p*-nitroanilides of benzyloxycarbonyl tetra- to hexa-peptides, provided that hydrophobic amino acid residues form new peptide bonds. The effectiveness of the enzyme also depends on the nature of adjacent amino acid residues. An aspartic proteinase with a characteristic gastricsin specificity pattern would be useful for the synthesis of middle-length peptides.

Key words: Gastricsin, peptide synthesis.

Pepsin, a typical aspartic proteinase, has been applied as a catalyst for peptide bond formation (Fruton, 1982; Jakubke, 1987). Presumably, the aspartic proteinase bovine gastricsin, could also be used for the same purpose. Gastricsin is homologous to pepsin but differs from pepsin in the pH optimum of its proteolytic activity which is shifted towards pH 2.8 (Yoon, 1970), and the favorable pH for its peptide bond formation is 4~5.

Characteristic features of gastricsin specificity are relevant for its synthetic application. The enzyme is sensitive to the length of the peptide to be split. Pentapeptides are the shortest substrates hydrolyzed by this enzyme, and hexapeptide esters are substantially better substrates for gastricsin than pentapeptide derivatives (Yoon, unpublished observation). All amino acids referred to in this paper belong to the L-series. Amino acid residues in the peptide substrates of gastricsin are referred to as suggested by Schechter and Berger (1967). The same approach was used for the residues in peptide components reacting in the presence of gastricsin. Hence, the residue that donates a carboxyl group to form a peptide bond in the course of peptide synthesis is termed P_1, the preceding residue P_2. The residue that donates an amino group is termed P'_1, the following residue P'_2 etc. For hydrolysis, a suitable substrate should contain hydrophobic residues at P_1 and P'_1, as well as at the P_3 and P'_3 positions. The use of gastricsin as a catalyst for peptide synthesis appears to be promising, although there are apparently no literature references to its application in peptide synthesis. Hence, this study was devoted to assessment of bovine gastricsin as a catalyst for peptide bond formation.

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Materials and Methods

Bovine gastricsin was purified from a bovine fourth stomach by the method of Yoon (1970). Absence of contamination by bovine pepsin was confirmed by SDS-PAGE (Foltmann *et al.*, 1985). Enzyme activity was tested by hemoglobin hydrolysis (Yoon, 1970). A standard gastricsin solution in 0.5 M acetate buffer, pH 5.1 (10 mg/ml), was prepared which, after a 10-fold dilution, showed an A_{280} value of 1.20.

Starting peptides and their derivatives were prepared at the laboratory of S. Aimoto, professor of Osaka University, Institute for Protein Research, Japan. Purity was checked by HPLC and amino acid analysis. Characterization of peptides was done by amino acid composition analysis using a Hitachi 835 amino acid analyzer after hydrolysis with 5.7 M HCl for 48 h at 110℃.

Reaction mixture composition and derivative purity were assessed by reverse-phase HPLC using a Gilson 704 liquid chromatograph, and an Ultrasphere ODS C-8 (for peptide esters) or a C-18 (for peptide *p*-nitroanilides) 4.5 mm×250 mm column. Solution A contained 5% (v/v) acetonitrile, 0.05 % (v/v) triethylamine, and 0.05 % (v/v) trifluoroacetic acid. Solution B contained 90% (v/v) acetonitrile, 0.05% (v/v) triethylamine, and 0.05% (v/v) trifluoroacetic acid. A linear gradient of solution B (from 40~80% in 15 min; program 1) was used for separation of peptide esters, which were detected at 215 nm. A linear gradient of solution B [from 30~60% in 25 min (program 2), or from 30~75% in 35 min (program 3)], with detection both at 215 and 315 nm, was used for separation of peptide *p*-nitroanilides. The rate of elution was 1.5 ml/min. No attempt was made to correct the peak areas for molar

Table 1. Synthesis of benzyloxycarbonyl-peptide esters catalyzed by bovine gastricsin at 5.1 and 37℃ for 24 h. Other reaction conditions were as indicated in the text

Peptides	Reaction time (h)	Yield (%)
Z-Val-Val-Phe↓Phe-Val-Val-OCH_3	24	68
Z-Val-Val-Phe↓Leu-Val-Val-OCH_3	24	61
Z-Val-Val-Phe↓Leu-Gly-Gly-OCH_3	48	4.5
Z-Val-Val-Phe↓Leu-Phe-OCH_3	72	40
Z-Val-Val-Phe↓Met-OCH_3	24	0
Z-Val-Val-Leu↓Phe-Val-Val-OCH_3	24	62
Z-Val-Val-Leu↓Leu-Val-Val-OCH_3	24	60

The peptide bonds formed are indicated by the arrows.

absorbance coefficients.

Synthesis of Z-Val-Val-Phe-Phe-Val-Val-OCH_3

Z-Val-Val-Phe-OH [25 mg (50 μM)] and H-Phe-Val-Val-OCH_3/HCl [22 mg (50 μM)] were dissolved in 200 μl of dimethylformamide (DMF) and 50 μl of a 1 M triethylamine solution in DMF, followed by 650 μl of 0.5 M acetate buffer, pH 5.1, and 100 μl of the standard gastricsin solution (1 mg of enzyme). After 24 h at 37℃ the precitpitate was collected by centrifugation, and subsequently washed with 3% (w/v) $NaHCO_3$ (4×1 ml), 1 ml of water, 0.1 M HCl (4×0.5 ml), then with water again, and was finally dried *in vacuo* over P_2O_5. The yield of Z-Val-Val-Phe-Phe-Val-Val-OCH_3 was 32.5 mg (68%). The preparation was substantially pure, according to amino acid analysis data and HPLC. Other benzyloxycarbonyl-peptide methyl esters (Table 1) were prepared in a similar manner.

Synthesis of Z-Val-Val-Phe-Leu-Val-Val-NH-Ph-NO_2

Z-Val-Val-Phe-OH [25 mg (50 μM)] and H-Leu-Val-Val-NH-Ph-NO_2 [24.6 mg (50 μM)] were dissolved in 200 μl of DMF, then 700 μl of 0.5 M acetate buffer, pH 5.1, was added followed by 100 μl of the standard gastricsin solution (1 mg of enzyme). The mixture was kept at 37℃ for 24 h with constant shaking. The precipitate was collected by centrifugation and washed as indicated above. After drying *in vacuo* over P_2O_5, 20 mg of Z-Val-Val-Phe-Leu-Val-Val-NH-Ph-NO_2 obtained (yield 41%). Other peptide *p*-nitroanilides (Table 2) were prepared using the same procedure.

Dependence of the hexapeptide derivative yield on pH

Aliquots (140 μl) of 0.5 M citrate buffer at various pH values (3.0, 4.0, 5.0, 5.5, 6.0) were added to a solution of 5 mg (10 μM) of Z-Val-Val-Phe-OH and 4.3 mg (10 μM) of H-Phe-Val-Val-OCH3/HCl in 40 μl of DMF and 10 μl of 1 M triethylamine solution in DMF, followed by 10 μl of gastricsin (0.1 mg) (enzyme/substrate ratio of 1 : 36000 in 0.5 M citrate buffer, pH 4.8). The mixtures were kept at 37℃ for 1 h with continual shaking, then 5 μl of triethylamine was added to stop the reaction. Dimethyl sulfoxide (DMSO) (500 μl) was added to the suspension to dissolve the precipitate. A 10 μl aliquot was diluted with 200 μl of methanol and this solution was applied to a HPLC column to assess the product content in the reaction mixture (Fig. 1). No reaction was observed at pH 2. This was established by addition of concentrated formic acid to a 0.5 M solution under pH meter control.

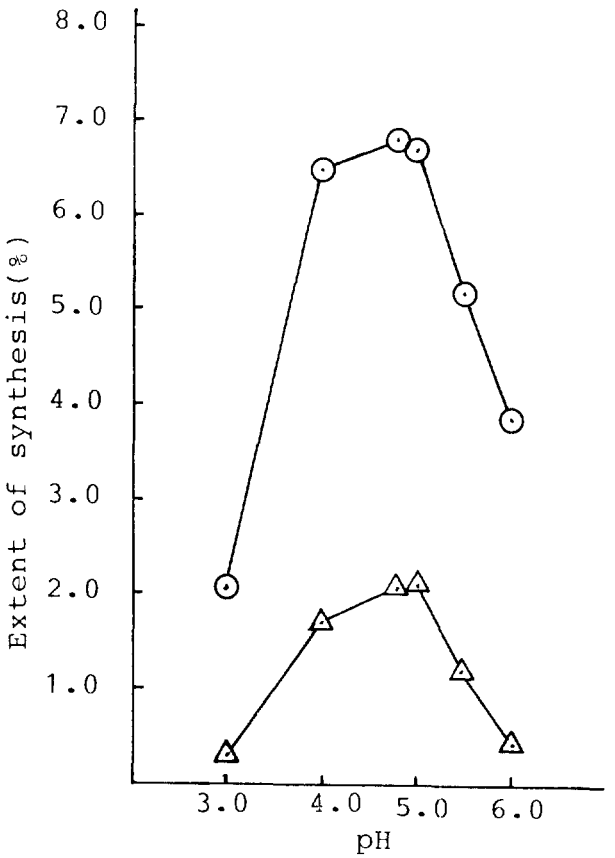

Fig. 1. Dependence upon pH of the yields of peptides obtained by gastricsin-catalyzed synthesis. The reactions were performed in 0.5 M citrate buffers containing 20% (v/v) DMF for 1 h at 37℃. The ratios of the product peak area to the sum of the acyl component and the product peak areas (measured at 215 nm) were expressed as percentages, which correspond to the yield of the product, and were plotted along the ordinate. (○—○), Z-Val-Val-Phe-Phe-Val-Val-OCH_3 synthesis. (△···△), Z-Val-Val-Phe-Leu-Val-Val-OCH_3 synthesis.

Results and Discussion

To verify gastricsin-catalyzed peptide bond formation it was decided to study gastricsin effectiveness in the condensation of the two protected tripeptides Z-Val-

Table 2. Synthesis of protected peptide *p*-nitroanilides catalyzed by bovine gastricsin at pH 5.1 and 37℃ for 24 h. Other reaction conditions were as indicated in the text

Peptides	Yield (%)
Z-Val-Val-Phe↓Leu-Val-Val-NH-Ph-NO_2	41
Z-Val-Val-Phe↓Val-Val-NH-Ph-NO_2	0
Z-Val-Val-Phe↓Val-Val-Phe-NH-Ph-NO_2	10
Z-Val-Val-Phe↓Leu-NH-Ph-NO_2	35
Z-Val-Val-Leu↓Leu-Val-Val-NH-Ph-NO_2	46

The peptide bonds formed are indicated by the arrows.

Table 3. Characteristics of peptides prepared by gastricsin-catalyzed synthesis

Peptide	HPLC program	Retention time (min)	Amino acid composition (nM)
Z-Val-Val-Phe-Phe-Val-Val-OCH_3	1	9.2	Val 14.5(4)*
			Phe 7.1(2)
Z-Val-Val-Phe-Leu-Val-Val-OCH_3	1	8.0	Val 16.2(4)
			Phe 4.3(1)
			Leu 4.5(1)
Z-Val-Val-Leu-Phe-Val-Val-OCH_3	1	7.1	Val 11.6(4)
			Phe 3.6(1)
			Leu 3.1(1)
Z-Val-Val-Leu-Leu-Val-Val-OCH_3	1	8.9	Val 14.5(4)
			Leu 7.6(2)
Z-Val-Val-Phe-Leu-Val-Val-NH-Ph-NO_2	2	11.8	Val 8.2(4)
			Phe 2.0(1)
			Leu 2.3(1)
Z-Val-Val-Leu-Leu-Val-Val-NH-Ph-NO_2	2	12.6	Val 14.5(4)
			Leu 8.1(2)
Z-Val-Val-Phe-Leu-NH-Ph-NO_2	3	30.2	Val 12.3(2)
			Phe 6.1(1)
			Leu 6.3(1)

*Values given in parentheses represent ratio of amino acid residues of peptide expressd to the nearest integer.

Val-Phe-OH and Z-Val-Val-Leu-OH, with the methyl esters and *p*-nitroanilides of several tri- and di-peptides and amino acids.

The first condensation reaction was:

$$\text{(1) Z-Val-Val-Xaa-OH} + \text{H-Y-OCH}_3 \xrightarrow{\text{Gastricsin}} \text{Z-Val-Val-Xaa-Y-OH}_3$$

where Xaa represents Phe or Leu, and Y represents Phe-Val-Val, Leu-Val-Val, Leu-Gly-Gly, Leu-Phe, or Met.

The second condensation reaction was:

$$\text{(2) Z-Val-Val-Xaa-OH} + \text{H-Y'-NHC}_6\text{H}_4\text{-NO}_2\text{(H-Y'-NH-Ph-NO}_2\text{)} \xrightarrow{\text{Gastricsin}} \text{Z-Val-Val-Xaa-Y'-NH-Ph-NO}_2$$

where Xaa represents Phe or Leu, and Y' represents Val-Val, Val-Val-Phe, Leu, or Leu-Val-Val.

These reactions were performed with equimolar quantities of both components at pH 5.1 in the presence of 20% (v/v) DMF, which was added to keep the reagents in solution. The molar ratio of enzyme to starting compounds was to 1 : 1800.

Gastricsin turned out to be an effective catalyst for peptide synthesis. Protected hexapeptides, like Z-Val-Val-Phe-Phe-Val-Val-OCH_3 (Table 1), were obtained with good yield similar to yield in the presence of swine pepsin. These results are consistent with the known data on gastricsin hydrolysis of middle-length peptides, characterized by preferential splitting of the peptide bonds that join two hydrophobic amino acid residues. Although the final yields of the peptides obtained by Phe-Phe and Phe-Leu bond formation were similar, the rates of synthesis were different. After the initial 50 min a yield of approx. 50% of Z-Val-Val-Phe-Phe-Val-Val-OCH_3 was attained, whereas only a 20% yield of Z-Val-Val-Phe-Leu-Val-Val-OCH_3 was achieved.

The pentapeptide derivative was obtained with only a moderate yield. Characteristically, the introduction of glycine instead of valine residues at P'_2 and P'_3 positions (when H-Leu-Gly-Gly-OCH_3 was used as an amino component) resulted in a sharp decrease in the yield, thus confirming the enzyme's sensitivity to the amino acid residues flanking the bond to be formed or hydrolyzed. Attempts to condense Z-Val-Val-Phe-OH with methionine methyl ester failed, although the Phe-Met bond is cleaved by gastricsin in its natural protein substrate. Methionine methyl ester, being devoid of an amido group, is too small to be effectively bound at the enzyme's S'_1 binding site. Gastricsin also failed to hydrolyze Phe-Met peptide bonds in short peptide derivatives (data not included). The *p*-nitroanilides of leucine and peptides with N-terminal leucine residues reacted with N-terminal protected tripeptides in the presence of gastricsin, resulting in satisfactory yields of peptide *p*-nitroanilides, whereas the derivatives of peptides with N-terminal valine residues were unacceptable as amino components in gastricsin-catalyzed condensations (Table 2).

Gasticsin, in contrast to pepsin, does not co-precipitate with the peptide formed in the course of the reaction. The latter phenomenon markedly hinders control of pepsin-catalyzed synthesis (Abdel Malak, 1992). Formation of protected hexapeptide esters catalyzed by gastricsin proceeds optimally at pH 4~5 (Fig. 1). In conclusion, gastricsin is suitable as a catalyst for peptide

synthesis (Table 3).

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