OBSERVATIONS ON THE SPECIFICITY OF THERMOLYSIN WITH SYNTHETIC PEPTIDES

Hiroshi Matsubara

Space Sciences Laboratory
University of California
Berkeley, California[1]

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It was found previously that thermolysin, a thermostable protease (Endo, 1962, 1965), hydrolysed preferentially peptide bonds involving the amino groups of hydrophobic amino acid residues with bulky side chains in several protein substrates (Matsubara, et al., 1965, 1966). In the present study, several synthetic peptides were tested as substrates for thermolysin. Only one type of peptide, exemplified by Cbz(Carbobenzoxy)-Gly-Pro-Leu-Ala-Pro, which has been used as a substrate for collagenase (Nagai, et al., 1960), has previously been found to be a good substrate for thermolysin (Ohta and Ogura, 1965).

In the searching for simpler substrates it was found that Cbz-Gly-L-Phe-amide and Cbz-Ala-L-Leu-hydrazide were readily hydrolysed by thermolysin. The present communication compares several synthetic peptides and presents a discussion of the structural requirement for their susceptibility to hydrolysis by this enzyme.

MATERIALS AND METHODS

Thermolysin was crystallised 4 times as described previously (Endo, 1962) and dissolved in 0.1 M Tris buffer, pH 8.0, with 0.002 M $CaCl_2$ to give 0.01 - 0.08 mg/ml solution. The peptides and their sources are listed in Table 1; they were used without further purification. They were dissolved in 1.5 ml of 0.05 M Tris buffer, pH 8.0, with 0.001 M $CaCl_2$ to give

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2 to 25 mM solutions and were digested with 0.3 ml of thermolysin solution at 37°-40°C. Aliquots of 0.2 ml of the reaction mixture were withdrawn at appropriate intervals over a two to four hour period and 2 drops of glacial acetic acid were added to terminate the reaction. With peptides containing no free amino group, the digests from the reaction mixtures were analysed by the usual ninhydrin method (Moore and Stein, 1948). The calibration was with L-leucine-amide or DL-phenylalanine under the same conditions. In the case of peptides with free amino groups, the hydrolysates were analysed on the amino acid analyser after dilution with pH 2.2 buffer (Spackman, et al., 1958). The hydrolysis of Cbz-Ala-L-Leu-hydrazide was estimated as follows: After passing the digests through Dowex 50 X 8 (H^+-form) column (0.6 X 3 cm), the resin was washed with water and the adsorbed materials were eluted with 1 N NH_4OH. They were dried and hydrolysed with 6 N HCl for 20 hr at 105°C. The analysis was performed as usual on the analyser. The leucine value obtained after subtraction of the alanine value was assumed to be the hydrolysis value. The bonds hydrolysed were identified by the analyser and by paper chromatography using BAW system (butanol:acetic acid:water = 200:30:75, v/v).

The initial velocity of the reaction was estimated from the curve obtained by the time course of the reaction.

RESULTS AND DISCUSSION

The reaction conditions and the initial velocity of the hydrolysis of various synthetic peptides by thermolysin are shown in Table 1. Thermolysin did not hydrolyse Gly-L-Phe, L-Pro-L-Ala, Gly-L-Asp or Gly-L-Pro. Gly-L-Phe-amide, which has no free α-carboxyl group, was not hydrolysed. However, Cbz-Gly-L-Phe, a peptide with no free amino group, was slightly hydrolysed and phenylalanine was liberated under certain conditions. When Cbz-Gly-L-Phe-amide, which has no free amino and carboxyl groups was used, thermolysin rapidly hydrolysed the peptide bond between glycine and phenylalanine. Similar results were obtained with Cbz-Gly-L-Leu and Cbz-

Ala-L-Leu-hydrazide. The latter substrate was hydrolysed very rapidly by thermolysin, and though a small amount of leucine was detected after the enzymatic reaction, the hydrolysis was principally at the Ala-Leu bond. In contrast, Cbz-Gly-Gly-amide was not hydrolysed to a measurable extent, suggesting that the specificity previously observed in protein substrates was also found in this investigation, namely, thermolysin attacks peptide bonds involving the amino groups of hydrophobic amino acid residues with bulky side chains (Matsubara, et al., 1966). A peptide, Cbz-Gly-L-Pro-L-Leu-Gly-L-Pro, was compared with those described above and the finding that this peptide was well utilized by the enzyme (Ohta and Ogura, 1965; Morihara and Tsuzuki, 1966) was confirmed.

From these results it is concluded that susceptibility to hydrolysis by thermolysin requires the absence of free amino and carboxyl groups from the immediate vicinity of the peptide bond to be hydrolyzed. Further-

TABLE I

Hydrolysis of Synthetic Peptides by Thermolysin

Peptide	Concentration of Peptide (mM)	Concentration of Enzyme (mg/ml)	Initial Velocity (μmole/ml/hr)
Gly-L-Phe[a)]	25	0.084	0
Gly-L-Phe-amide[b)]	25	0.084	0
Cbz-Gly-L-Phe[c)]	23	0.085	0.025
Cbz-Gly-L-Phe-amide[a)]	1.7	0.014	1.6
Cbz-Gly-L-Leu[a)]	24	0.084	0.030
Cbz-Ala-L-Leu-Hydrazide[a)]	1.7	0.020	$\sim$ 2.6
Cbz-Gly-Gly-amide[a)]	1.6	0.017	$\sim$ 0
Cbz-Gly-L-Pro-L-Leu-Gly-L-Pro[c)]	1.7	0.017	$>$ 0.9

a) New England Corp., Boston, Mass.

b) Peptide Center, The Institute for Protein Research, Osaka University, Japan

c) Sigma Chem. Co., St. Louis, Mo.

more, for susceptibility to the enzyme, the side chain of the amino acid whose amino group is involved in the bond should be hydrophobic and bulky.

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