EFFECT OF PROLINE RESIDUE ON THE HYDROLYSIS OF SUBSTRATES BY THERMOLYSIN

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Thermolysin, a proteolytic enzyme of a thermophilic bacterial origin, hydrolyses the peptide bonds of proteins and peptides at the amino sites of hydrophobic amino acids with bulky side chains (Matsubara et al., 1965; Matsubara, 1966; Matsubara et al., 1966; Morihara and Tsuzuki, 1966; Morihara and Ebata, 1967; Morihara et al., 1968). Thermolysin was found to be very useful in the study of protein sequences and it has successfully been used by many workers in sequence studies.

In an earlier report it was noted that there was a peptide bond in beef cytochrome c resistant to hydrolysis by thermolysin (Matsubara, 1967). The amino acid sequence of the region involved was -Lys-Tyr-Ile-Pro-Gly-(see Table I). A similar peptide bond resistant to thermolysin digestion was found in spinach ferredoxin during the study of its sequence (Matsubara et al., 1967; Matsubara and Sasaki, 1968). Recently, thermolysin was used to hydrolyse azurin from Ps. aeruginosa and it was suggested that the enzyme failed to hydrolyse peptide bonds at the amino sites of hydrophobic amino acids if a proline residue was present at their carboxyl sites (Ambler and Meadway, 1968).

We synthesized several peptides and their derivatives to examine the influence of proline residue on hydrolysis. Thermolysin has no ability to hydrolyse the peptide bonds at the amino sites of hydrophobic amino acid residues having a proline residue at their carboxyl sites, regardless

of the presence or absence of a second residue attached to the carboxyl group of proline.

EXPERIMENTAL

Materials: Thermolysin, a gift of Dr. S. Endo, was recrystallized three times. The enzyme solution was made and stored as described (Matsubara et al., 1966). Peptides, Leu-Asp-Leu-Pro-Tyr and Val-Thr-Leu-Lys, were obtained from Scenedesmus ferredoxin (Sugeno and Matsubara, 1968). The peptide, Leu-Asp-Leu-Pro, was prepared after complete digestion of the peptide, Leu-Asp-Leu-Pro-Tyr, by carboxypeptidase A (Worthington Biochem. Corp.) and purification by paper chromatography. Gly-L-Leu-Gly was purchased from Mann Research Lab., Inc. Gly-L-Leu-L-Pro, Gly-L-Leu-L-Pro-Gly, and Gly-L-Leu-D-Pro were synthesized by the solid phase method of Merrifield essentially as described by Young et al (1967). The materials, t-BOC-Gly-resin ester, t-BOC-L-Pro-resin ester, t-BOC-L-Leu, t-BOC-L-Pro and t-BOC-Gly, were purchased from Schwarz BioResearch, Inc. t-BOC-D-Pro was a generous gift of Dr. J. M. Stewart. All peptides synthesized showed satisfactory composition and purity. Dinitrophenyl (DNP) derivatives of the peptides were prepared according to the method of Fraenkel-Conrat et al (1956).

Enzymatic Hydrolysis of Peptides and Identification of Hydrolysis Products: Various peptides (0.5 - 1.0 μmole) dissolved in 0.2 - 0.4 ml of 0.05 M Tris-HCl buffer at pH 8.0 containing 0.01 M $CaCl_2$ were digested with 0.05 - 0.2 mg of thermolysin for 16 hours at 40°. The reaction mixture was chromatographed on paper with butanol-acetic acid-water (100:15:37.5, v/v). Yellow spots of DNP-peptides or -amino acids, and ninhydrin positive spots were analysed with the amino acid analyser, Spinco 120C.

RESULTS AND DISCUSSION

Examples in the Literature: The use of thermolysin or other enzymes having similar specificity was reported by various investigators. Pertinent findings are in Table 1. Thermolysin and B. subtilis neutral proteases (McConn et al., 1964; Feder, 1967) failed to hydrolyse peptide bonds usually sensitive to the enzymes if a proline residue occupied the position at the carboxyl site of the

Table I

Peptide Sequences with Proline Residue Preventing Hydrolysis by Thermolysin and *B. subtilis* Neutral Protease

Enzyme	Sequence	Material	Reference
Thermolysin	-Ile-Asp-Leu-Pro-Tyr-	Spinach ferredoxin	Matsubara et al., 1967
		Taro ferredoxin	Rao and Matsubara, 1968
	-Leu-Asp-Leu-Pro-Tyr-	*Scenedesmus* ferredoxin	Sugeno and Matsubara, 1968
	-Lys-Tyr-Ile-Pro-Gly-	Beef cytochrome *c*	Matsubara et al., 1965 Matsubara, 1967
	-Gly-Asn-Leu-Pro-Lys-	*Ps. aeruginosa* azurin	Ambler and Meadway, 1968
	-Cys-Thr-Phe-Pro-Gly-		
	-Ala-Met-Val-Pro-Met-	E. coli. tryptophanase	Kagamiyama, 1968
	-Asp-Pro-Ile-Pro-Ser	MS2 coat protein	Lin et al., 1967 Wallis and Naughton, 1968
	-Thr-Glu-Ile-Pro-Asp-	E. coli acyl carrier protein	Vanaman et al., 1968
B. subtilis neutral protease	-Val-Gly-Ala-Pro-Val-	*C. pasteurianum* ferredoxin	Benson and Yasunobu, 1968

*Arrows show peptide bonds sensitive to the proteases unless a proline residue occupies the position shown.

sensitive hydrophobic amino acid residues. The common sequence which prevents the hydrolytic action of these enzymes is -X-Pro-, where X represents an hydrophobic amino acid residue whose amino site would be the sensitive bond unless there is an adjoining proline residue. This characteristic should enable certain unknown sequences in peptides to be predicted when these enzymes are used.

Hydrolysis of Proline Peptides by Thermolysin: It is unknown whether or not a residue adjoining the carboxyl site of the proline residue is necessary for an inhibitory effect on the hydrolysis of the peptide bond at the amino site of a hydrophobic residue preceding the proline residue. Various peptides were prepared and reacted with the enzyme. The results are shown in Table II. In the presence of a proline residue thermolysin could not hydrolyse the sensitive bond regardless of the presence or absence of a residue attached through the carboxyl group of proline, and regardless of whether the proline was D or L.

We have no definite explanation for this property of thermolysin and B. subtilis proteases, but perhaps the active sites of these enzymes are fairly rigid, and a steric hindrance caused by the proline residue, which makes a sharp turn in the peptide chain (Schellman and Schellman, 1964), prevents the hydrolysis of the bonds.

Table II

Hydrolysis of Various Peptides by Thermolysin

Peptide	Hydrolysis(%)	Products
Leu-Asp-Leu-Pro-Tyr	0	None
Leu-Asp-Leu-Pro	0	None
di-DNP- Leu-Asp-Leu-Pro-Tyr	0	None
DNP- Leu-Asp-Leu-Pro	0	None
di-DNP- Val-Thr-Leu-Lys	100	DNP-Val-Thr;Leu-DNP-Lys
DNP- Gly-Leu-Gly	60	DNP-Gly;Leu-Gly
DNP- Gly-Leu-Pro	0	None
DNP- Gly-Leu-Pro-Gly	0	None
DNP- Gly-Leu-D-Pro	0	None

It is interesting to note that these enzymes also failed to hydrolyze tryptophan peptides, Cbz-Leu-Trp-NH_2 (Feder, 1967) and DNP-Gly-Trp-Gly (Matsubara, 1969). This may also suggest rigidity of the active sites of the enzymes. A recent study of various bacterial neutral proteases suggested that they had a large active site covering at least 21 Å and several subsites (Morihara and Oka, 1968). Accordingly, our results give another scope of the size of active area of the enzyme.

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