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ACYL AND AMINO INTERMEDIATES IN REACTIONS CATALYZED BY THERMOLYSIN

K. Morihara, H. Tsuzuki and T. Oka

Shionogi Research Laboratory, Shionogi & Co., Ltd.,

Fukushima-ku, Osaka 553, Japan

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SUMMARY

Thermolysin showed peculiar transpeptidation reactions. Leu-Leu and/or Leu-Leu-Leu were produced at ca. pH 7 from Leu-Leu-NH_2 and Cbz-Leu-Leu. Isotope experiments indicated that the transpeptidation products did not use leucine released from the substrates as an acceptor. With Leu-Trp-Met, Leu-Leu, Leu-Leu-Leu and Met-Met were produced as transpeptidation products. A comparative study was done with α-chymotrypsin and pepsin. These results would indicate that thermolysin catalyzed reactions proceed via both acyl and amino intermediates depending upon the substrates, which has been proposed for the mechanism of pepsin. This may also be true in some cases for chymotrypsin and other proteases, which have been known as enzymes of the acyl-enzyme mechanism.

The acyl-enzyme mechanism for serine proteases is well established (1). Recently, a mechanism of catalysis by thermolysin has been proposed from its X-ray study (2) in which Glu-143, acting as a general base, promotes the attack of a water molecule on the carbonyl carbon of the scissile peptide bond which has already been somewhat depolarized by Zn^{2+}, and His-231 donates a proton to the peptide nitrogen forming a tetrahedral intermediate. However, no information is available concerning the pathway of catalysis via the acyl or amino intermediate.

Wang and Hofmann (3) have shown that pig pepsin catalyzes transpeptidation reactions which proceed via acyl and amino intermediates depending upon the substrates. Leu-Leu and/or

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Leu-Leu-Leu were produced from the substrate Leu-Leu-NH_2 (acyl-transfer type) and from Cbz-Phe-Leu (amino-transfer type), in which leucine-CO-E (acyl enzyme) or E-NH-leucine (amino enzyme) was postulated to form Leu-Leu-Leu-NH_2 and Leu-Leu-Leu-Leu-NH_2 or Cbz-Phe-Leu-Leu and Cbz-Phe-Leu-Leu-Leu, which would immediately be hydrolyzed into the above-mentioned products. With either Leu-Trp-Met (3) or [^{14}C]Leu-Tyr-[^{3}H]Leu (4) as a substrate, both acyl and amino transpeptidation products were formed.

In the present study, undertaken to investigate the pathway of reactions catalyzed by thermolysin, we determined the transpeptidation reactions using Hofmann's substrates.

MATERIALS AND METHODS

Thermolysin (crystals) and α-chymotrypsin (crystallized three times) were supplied from Daiwa Kasei Co., Osaka, Japan and Worthington Biochemical Corp., Freehold, N.J., U.S.A., respectively.

L-Leucine, L-methionine, Cbz-Gly-Leu and Cbz-Leu-Leu were supplied from The Protein Research Foundation, Minoh, Japan. Leu-Leu, Leu-Leu-Leu, Met-Met, Cbz-Phe-Leu and Leu-Leu-NH_2 were purchased from Vega-Fox Biochemicals, Tucson, Az., U.S.A. Leu-Trp-Met was obtained from Research Plus Laboratories Inc., Denville, N.J., U.S.A. [^{14}C]Leucine (354 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Abbreviated designations of amino acid derivatives, peptides, or their derivatives conform to the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature (1972). Except when specified, the constituent amino acids were all of the L-configuration.

The quantitative determination of amino acids and oligopeptides in the reaction mixture was made using an amino acid analyzer (Japan Electron Optics Laboratory, Model JLC-3) according to the method of Wang and Hofmann (3). The mixture of Leu-NH_2 and Leu-Leu-NH_2 was separated on a column (0.8 x 15 cm) of chromosorv #2611 resin using 0.7 M sodium citrate, pH 5.28, at 55°C. The flow rate was 70 ml/h. The elution time of Leu-NH_2 and Leu-Leu-NH_2 was 193 and 225 min, respectively. The mixture of the other reaction products was separated on a column (0.8 x 50 cm) of chromosorv #3105 resin at 60°C. The buffers were 0.2 M sodium citrate, pH 4.25, and 0.35 M sodium citrate, pH 5.28, and the buffer change occurred at 70 min. The elution time of L-methionine, L-leucine, Met-

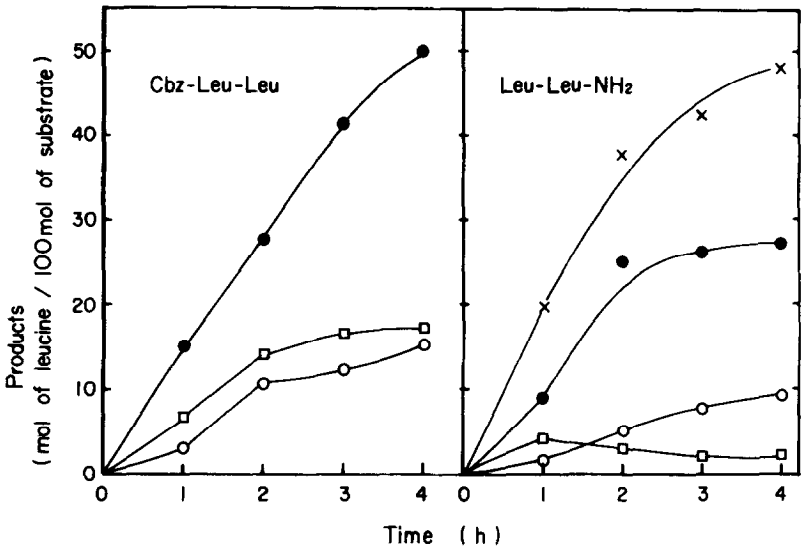

Fig. 1. Progress curve of cleavage of Cbz-Leu-Leu (2 mM) and Leu-Leu-NH_2 (2 mM) by thermolysin (1 mg/ml) at pH 7.1 and at 37°C.

The products were analyzed by an amino acid analyzer. O, Leucine; ●, Leu-Leu; □ Leu-Leu-Leu; and X, Leu-NH_2.

Met, Leu-Leu and Leu-Leu-Leu were 74, 82, 142, 152 and 164 min, respectively.

The reaction products from substrates were separated and identified by high-voltage electrophoresis in pyridine-acetic acid-water (1:20:279, by volume) at pH 3.5 and 25 V/cm for 120 min using authentic compounds for comparison. For the analysis of reaction mixtures containing [^{14}C]leucine, guide strips were stained and the unstained part of the electrophoretogram was cut into 2 cm-wide bands. These were put into scintillation bottles containing p-bis-(o-methylstyryl)benzene (0.05%) and 2,5-diphenyloxazole (0.5%) in toluene, and their radioactivity was measured in a Beckmann DPM-100 liquid-scintillation counter.

RESULTS AND DISCUSSION

The progress of the action of thermolysin on Cbz-Leu-Leu and Leu-Leu-NH_2 is shown in Fig. 1. The C- or N-terminal leucine in either peptide is converted into the products, leucine, Leu-Leu and Leu-Leu-Leu. In the case of Leu-Leu-NH_2, the sum of the products is almost equal to Leu-NH_2, which

Table I. Effect of pH on the hydrolysis and transpeptidation reactions catalyzed by thermolysin

The reaction mixture contained 2 mM substrate, thermolysin (1 mg/ml), 0.1 M Tris-buffer (various pH values as indicated in the table) and 1 mM $CaCl_2$, which was kept at 37°C for 2 h.

Substrate	pH	Leucine	Leu-Leu	Leu-Leu-Leu	Leu-NH_2
		(mol of leucine/100 mol of substrate)			
Cbz-Leu-Leu	6.2	26	17	9	
	7.1	10	28	14	
	7.8	5	15	7	
	9.3	0.4	2	2	
Leu-Leu-NH_2	6.2	3	19	1	33
	7.1	5	25	3	38
	7.8	3	11	4	30
	9.3	1	1	0	4

supports this proposal. The optimum pH for transpeptidation is ca. 7, as shown in Table I.

The transpeptidation reactions of thermolysin were compared with those of α-chymotrypsin and pepsin, as seen in Table II. The data of pig pepsin were deduced from the study of Wang and Hofmann (3). In thermolysin catalyzed reactions of Cbz-AA-Leu, preferential hydrolysis instead of transpeptidation occurs when AA is L-phenylalanine, while the reverse is the case when AA is L-leucine or glycine. Similar case can be observed with α-chymotrypsin, although the reaction with Cbz-AA-Leu is very slow in comparison with that of thermolysin. α-Chymotrypsin can catalyze transpeptidation reaction with Leu-NH_2, as well as with Leu-Leu-NH_2; the velocity of Leu-Leu-NH_2 is comparable to that of thermolysin. The transpeptidation activity of pepsin roughly corresponds to that of

Table II. Comparison of transpeptidation reactions catalyzed by thermolysin with those by α-chymotrypsin and pepsin

The reaction mixture contained 2 mM substrate, 0.1 M Tris-buffer (pH 7.1), 1 mM $CaCl_2$, and thermolysin (1 mg/ml) or α-chymotrypsin (2 mg/ml) was kept at 37°C. In the case of Leu-NH_2, the concentration of α-chymotrypsin was 10 mg/ml. The reaction time is shown in the table.

Enzyme	Substrate	Reaction time (h)	Leucine	Leu-Leu	Leu-Leu-Leu	Leu-NH_2
			(mol of leucine/100 mol of substrate)			
Thermolysin	Cbz-Phe-Leu	2	74	4	1.5	
	Cbz-Leu-Leu	2	10	28	14	
	Cbz-Gly-Leu	2	12	36	3	
	Leu-Leu-NH_2	2	5	25	3	38
α-Chymotrypsin	Cbz-Phe-Leu	20	53	5	0	
	Cbz-Leu-Leu	20	2	15	0	
	Leu-Leu-NH_2	4	0.8	7	15	20
	Leu-NH_2	20	15	18	2	60
Pepsin	Cbz-Phe-Leu[a]	2	2	3	17	
		4	5	12	32	
	Leu-Leu-NH_2[b]	2	2	0.5	1	6
		4	4	2	2.5	10

[a] Reference (3): [S] = 2 mM, [E] = 1 mg/ml, pH 4.7, 37°C.

[b] Reference (3): [S] = 4 mM, [E] = 2 mg/ml, pH 3.4, 37°C.

thermolysin. Similar results were observed in these enzymes with Leu-Trp-Met as substrate, as seen in Table III.

Transpeptidation by thermolysin and α-chymotrypsin was further determined in the presence of [^{14}C]leucine in the reaction mixture. The results are summarized in Table IV, which indicates that the transpeptidation does not occur using leucine released from the substrates as an acceptor.

The mechanism of acid proteases (5), which is based on the crystal structure of penicillopepsin, is similar in many respects to that of thermolysin (2). Transpeptidation is a

Table III. Transpeptidation reactions of Leu-Trp-Met by thermolysin, α-chymotrypsin and pepsin

The reaction mixture contained 2 mM Leu-Trp-Met, 0.1 M Tris-buffer (pH 7.1), 1 mM $CaCl_2$, and thermolysin (1 mg/ml) or α-chymotrypsin (2 mg/ml), which was kept at 37°C. The reaction time of thermolysin or α-chymotrypsin was 2 or 20 hours, respectively.

	Leucine	Leu-Leu	Leu-Leu-Leu	Methionine	Met-Met	Met-Met-Met
	(mol of leucine/100 mol of substrate)					
Thermolysin	3	28	8	6	25	-
α-Chymotrypsin	2	3	2	71	3	-
Pepsin[a]	3	12	36	2	4	9

[a] Reference (3): [S] = 2 mM, [E] = 0.6 mg/ml, pH 3.4, 37°C, 2 h.

Table IV. Thermolysin or α-chymotrypsin catalyzed transpeptidation in the presence of [^{14}C]leucine

Each reaction mixture (0.5 ml) contained 0.2 mM [^{14}C]-leucine (1 μCi). The other experimental conditions are described in Table II.

Enzyme	Reactions	Product radioactivity (c.p.m.) Leucine	Leu-Leu and/or Leu-Leu-Leu	Leu-NH_2
Background		182	141	84
Thermolysin	Cbz-Leu-Leu + [^{14}C]leucine	73,580	139	76
	Leu-Leu-NH_2 + [^{14}C]leucine	60,382	186	89
Chymotrypsin	Cbz-Leu-Leu + [^{14}C]leucine	74,449	143	103
	Leu-NH_2 + [^{14}C]leucine	71,581	136	70

phenomenon peculiar to the acid proteases, and proceeds via both acyl and amino intermediates (2). This was also observed in this study with thermolysin. In the case of pepsin, a general base mechanism of hydrolysis is proposed (5) assuming the existence of a ternary complex of enzyme, acyl fragment and amino fragment after cleavage of the susceptible peptide bond in place of the involvement of either an anhydride or an amino-enzyme intermediate, with the order of product release being determined by the relative affinity of the acyl or amino moiety to their respective binding sites. This may be true for thermolysin and in some cases for chymotrypsin and other proteases which have been known as enzymes of the acyl-enzyme mechanism.

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