Specificity of Proteinase K from *Tritirachium album* Limber for Synthetic Peptides

Kazuyuki MORIHARA and Hiroshige TSUZUKI

Shionogi Research Laboratory, Shionogi & Co., Ltd.,
Fukushima-ku, Osaka 553

Received March 10, 1975

The specificity of proteinase K from *Tritirachium album* Limber was determined using various synthetic peptide substrates. The esterase activity against N-acylated amino acid esters indicated that the enzyme is primarily specific against aromatic or hydrophobic amino acid residues at the carboxyl side of the splitting point. Secondary interaction for hydrolysis was also studied using peptide esters or others, which showed that the enzyme activity is markedly promoted by elongating the peptide chain to the N-terminal from the splitting point. Thus, peptide chloromethyl ketone derivatives such as Cbz-Ala-Gly-PheCH₂Cl inactivated the enzyme activity markedly.

Ebeling *et al.*¹⁾ found a strain of the mold *Tritirachium album* which can grow on native keratin as the sole carbohydrate and nitrogen source. The organism secreted a highly active mixture of proteases along with an aminopeptidase into the culture medium. The main protease was named "proteinase K" for its keratin-hydrolyzing activity, and the enzyme was purified to a homogeneous state and some general properties were clarified.¹⁾ The study indicated that proteinase K is a typical serine alkaline proteinase. We compared^{2~4)} the specificity of various serine alkaline proteinases of microbial origin and found that they show similar specificity with each other against oxidized insulin B chain or synthetic peptide substrates. The present study was undertaken to confirm this specificity of proteinase K using various synthetic peptides as substrates.

MATERIALS AND METHODS

Enzyme. A highly purified preparation of proteinase K was kindly supplied by E. Merck (Darmstadt).

Substrates and inhibitors. Ac-Phe-OEt, Ac-Tyr-OEt, Ac-Trp-OEt, and Bz-Arg-OEt were supplied by The Protein Research Foundation, Osaka. Ac-Val-OMe and Cbz-Ala-Tyr-NH₂ were obtained from Cyclo Chemical Corporation, Los Angeles. Ac-Gly-OEt, Ac-Ala-OMe, Ac-Leu-OMe, and Cbz-Lys-OMe were synthesized as described previously.^{2,5)} Cbz-AA-Lys-OMe (AA=glycine, D- and L-alanine, L-leucine, and L-phenylalanine), Cbz-(Ala)_m-Lys-OMe (m=1 and 2), Ac-(Ala)₃-OMe, and Ac-(Ala)₂-Phe-OMe were synthesized according to a method described previously.^{4,6)} Various peptides such as Cbz-Ala-Gly-Tyr-NH₂, Cbz-Gly-Leu-Gly, Cbz-Gly-Leu-(Gly)₂, and Cbz-Gly-Leu-(Gly)₂-Ala were prepared as described previously.^{3,6)} Chloromethyl ketone derivatives, Cbz-PheCH₂Cl, Cbz-Ala-PheCH₂Cl, and Cbz-Ala-Gly-PheCH₂Cl, were synthesized according to the method given in the previous paper.⁷⁾

Determination of enzymatic activity. Esterase activity was determined using a Radiometer Type TTT1 pH-stat equipped with a syringe buret, a Type SBR2c recorder, and a thermostatically controlled reaction vessel (30°C). Reactions were carried out in 0.1 M KCl at pH 7.5, with 0.05 N NaOH as the titrant. In all cases, the data were in accordance with Michaelis-Menten kinetics over the range of substrate concentration (~10 mM) employed: five to eight runs were performed for each determination of *K_m* and *K_{cat}* (V per molar equivalent of enzyme); the enzyme concentration was chosen to give reliable data for the

Abbreviations used are as follows: TPCK, tosyl L-phenylalanine chloromethyl ketone; TLCK, tosyl L-lysine chloromethyl ketone. Abbreviated designations of amino acid derivatives, peptides, and their derivatives obey the tentative rules of the IUPAC-IUB Commission on Biochemical Nomenclature. Except where specified, the constituent amino acids were all of L-configuration.

initial rate of hydrolysis. In the calculation of k_{cat} , the molecular weight of proteinase K was taken as 18,500.¹⁾

Either peptidase or amidase activity was determined as follows: A reaction mixture (1 or 5 ml) containing 2 mM peptide, 0.1 mg of enzyme (per ml), and 0.05 M Tris buffer (pH 7.4) in the presence (peptide amides) or absence of 4% dimethylformamide was incubated at 40°C. After 30 min or 20 hr reaction, 0.1 ml (or 1 ml) of the reaction mixture was withdrawn and put into a test tube which contained 1 ml (or 2 ml) of 0.5 M citrate buffer (pH 5.0), and cooled in an ice-bath to prevent further hydrolysis. The extent of hydrolysis was measured by the ninhydrin method of Yemm and Cocking.⁸⁾ The color yields of the compounds by the ninhydrin method based on L-leucine as 100% were described in the preceding paper.⁹⁾ The sites of action of enzymes in the substrates were determined by paper chromatography of the hydrolyzates, or by the standard DNP method. The release of ammonia from substrates was examined using Conway's apparatus.

Inhibition study. Inactivation by various chloromethyl ketone derivatives was determined as follows: The reaction mixture (5 ml) containing 0.05 M Tris

buffer (pH 7.0), 100 μ M inhibitor, 5 μ M enzyme, and 10% dioxane was kept at 40°C. At various intervals, the remaining activity was determined using Ac-Tyr-OEt as substrate. The esterase activity was followed in a pH-stat, at pH 8.0 and 30°C, as described above. The initial velocity was calculated from the slope of the plot of base consumption against time. Since the inactivation reactions were all pseudo-first order, the k (sec^{-1}) values of the reagents were calculated.

RESULTS AND DISCUSSION

The esterase activity against N-acylated amino acid esters was determined as shown in Table I, in which a comparison is made with the serine alkaline proteinase from *Aspergillus oryzae*. The table indicates that proteinase K is specific against aromatic or hydrophobic amino acid residues whose carbonyl group contains the susceptible bond, as has been suggested by Ebeling *et al.*¹⁾ The specificity is similar to that of the enzyme from *A. oryzae* except that the proteolytic coefficient against

TABLE I. ESTERASE ACTIVITY AGAINST N-ACYLATED AMINO ACID ESTERS

Substrate			Proteinase K		Alkaline proteinase ^{a)} from <i>A. oryzae</i>	
			K_m (mM)	k_{cat}/K_m ($\text{sec}^{-1} \text{mM}^{-1}$)	K_m (mM)	k_{cat}/K_m ($\text{sec}^{-1} \text{mM}^{-1}$)
Ac	Gly	OEt	670	0.003		0
Ac	Ala	OMe	45	1.5	91	0.6
Ac	Val	OMe	26	0.1		0
Ac	Leu	OMe	30	4.5	15	1.8
Ac	Phe	OEt	12	8.1	12	9.4
Ac	Tyr	OEt	8.8	10.7	23	6.1
Ac	Trp	OEt	5.6	33.4	18	2.9
Cbz	Lys	OMe	21	1.3	(31)	(5.6) ^{b)}
Bz	Arg	OEt	3.3	0.3	7.5	13.8

^{a)} Data from Morihara and Tsuzuki.²⁾

^{b)} Data of the serine alkaline proteinase from *Aspergillus sojae*.⁴⁾

TABLE II. ESTERASE ACTIVITY AGAINST N-ACYLATED PEPTIDE ESTERS

Substrate				Proteinase K		Alkaline proteinase ^{a)} from <i>A. sojae</i>	
				K_m (mM)	k_{cat}/K_m ($\text{sec}^{-1} \text{mM}^{-1}$)	K_m (mM)	k_{cat}/K_m ($\text{sec}^{-1} \text{mM}^{-1}$)
	Cbz	Lys	OMe	21	1.3	31	5.6
	Cbz	Ala	Lys	3	57.3	13	130
	Cbz	(Ala) ₂	Lys	0.9	689	1.5	1940
	Ac	(Ala) ₂	Phe	0.8	425	6.7	391
	Ac	(Ala) ₂	Ala	1.0	290	32	116

^{a)} Data from Morihara *et al.*⁴⁾

TABLE III. ESTERASE ACTIVITY AGAINST Cbz-AA-Lys-OMe

Substrate				Proteinase K		Alkaline proteinase ^{a)} from <i>A. sojae</i>	
				<i>K_m</i> (mM)	<i>k_{cat}/K_m</i> (sec ⁻¹ mM ⁻¹)	<i>K_m</i> (mM)	<i>k_{cat}/K_m</i> (sec ⁻¹ mM ⁻¹)
Cbz	Gly	Lys	OMe	19	3.8	66	20
Cbz	Ala	Lys	OMe	3	57.3	13	130
Cbz	D-Ala	Lys	OMe	20	0.1	50	0.5
Cbz	Leu	Lys	OMe	9	12.9	0.7	3060
Cbz	Phe	Lys	OMe	3	5.7	0.5	1060

^{a)} Data from Morihara *et al.*⁴⁾

TABLE IV. AMIDASE OR PEPTIDASE ACTIVITY OF PROTEINASE K

The reaction mixture containing 2 mM substrate, 0.1 mg of enzyme (per ml), and 0.05 M Tris-buffer (pH 7.4) in the presence (peptide amides) or absence of 4% dimethylformamide was kept at 40°C.

Substrate					Proteinase K		Alkaline proteinase ^{a)} from <i>A. oryzae</i>		Subtilisin BPN' ^{a)}	
					0.5 hr	20 hr	0.5 hr	20 hr	0.5 hr	20 hr
					(% of hydrolysis)					
			↓							
	Cbz	Tyr	NH ₂		—		0		< 5	
	Cbz	Ala	Tyr	NH ₂	18	65	0	26	100	
Cbz	Ala	Gly	Tyr	NH ₂	36	100	0	100	100	
			↓							
Cbz	Gly	Leu	Gly			0	0		0	
Cbz	Gly	Leu	(Gly) ₂		32	100	5	7	93	
Cbz	Gly	Leu	(Gly) ₂ Ala		54	100	15	95	11	95

^{a)} Data from Morihara *et al.*

Bz-Arg-OEt is below 1/40 that of the *Aspergillus* enzyme. The high esterase activity of the latter enzyme against Bz-Arg-OEt may not usually show its specificity against basic amino acid residues, but may be ascribed to the amino-blocking residue Bz as has been pointed out by Ku *et al.*¹⁰⁾

Table II gives the esterase activity against N-acylated peptide esters, and compared it with that of the serine alkaline proteinase from *Aspergillus sojae*. The esterase activity against Cbz-Lys-OMe is markedly promoted by more elongation of the peptide chain with alanine residue, a similar case being observed with *A. sojae* enzyme. Both enzymes thus show markedly high esterase activity against Ac-(Ala)₂-Phe-OMe and Ac-(Ala)₃-OMe, as well as Cbz-(Ala)₂-Lys-OMe.

The P₂-specificity* was determined using

* The amino acid residues in peptide substrates are numbered according to the system of Schechter and Berger.¹¹⁾

Cbz-AA-Lys-OMe (AA = various amino acid residues) as substrates. The result is shown in Table III, which also gives the data of the *A. sojae* enzyme for comparison. It indicates that alanine is most effective similar to the case of subtilisin BPN'.⁴⁾ This is not seen with the *A. sojae* enzyme, whose esterase activity is markedly promoted when hydrophobic or bulky amino acid residues occupy the P₂ position. The marked difference in the proteolytic coefficients against Bz-Arg-OEt between proteinase K and *A. oryzae* enzyme may thus be ascribed to the P₂-specificity corresponding to Bz.

A comparative study^{3,4)} of the specificity of various serine alkaline proteinases of microbial origin has indicated that mold enzymes such as those obtained from *A. oryzae*, *A. melleus*, and *A. sojae* do not show any amidase activity on peptide amide substrates, while bacterial enzymes such as subtilisins show considerable activity. Furthermore, it has

been shown³⁾ that the mold enzymes essentially require the presence of three amino acid residues at the C-terminal from the splitting point for hydrolysis of the peptide substrates, while bacterial enzymes require the presence of two amino acid residues. The result given in Table IV indicates that proteinase K is more similar to bacterial than mold enzymes on this point.

Proteinase K is insensitive to both TPCK and TLCK, as seen similarly with usual serine alkaline proteinases from microorganisms. The latter enzymes are, however, sensitive to peptide chloromethyl ketone derivatives such as Cbz-Ala-PheCH₂Cl and Cbz-Ala-Gly-PheCH₂Cl.^{3,4)} Proteinase K is also markedly sensitive to them, especially to the tripeptide chloromethyl ketone, as seen in Table V.

The present kinetic study using synthetic peptide substrates indicates that proteinase K

shows specificity similar to those of usual serine alkaline proteinases of microbial origin not only in primary specificity but also in the effect of secondary interaction. Interestingly, the effect of neighboring amino acid residues (secondary interaction) on hydrolysis is more similar to that of bacterial rather than mold enzymes.

Acknowledgements. The authors are greatly indebted to Dr. H. Lang of E. Merck (Darmstadt) for his kind gift of highly purified proteinase K.

REFERENCES

- 1) W. Ebeling, N. Hennrich, M. Klockow, H. Metz, H. D. Orth and H. Lang, *Eur. J. Biochem.*, **47**, 91 (1974).
- 2) K. Morihara and H. Tsuzuki, *Arch. Biochem. Biophys.*, **129**, 620 (1969).
- 3) K. Morihara, T. Oka and H. Tsuzuki, *ibid.*, **146**, 297 (1971).
- 4) K. Morihara, T. Oka and H. Tsuzuki, *ibid.*, **165**, 72 (1974).
- 5) K. Morihara and T. Oka, *ibid.*, **156**, 764 (1973).
- 6) K. Morihara, T. Oka and H. Tsuzuki, *ibid.*, **132**, 489 (1969).
- 7) K. Morihara and T. Oka, *ibid.*, **138**, 526 (1970).
- 8) E. W. Yemm and E. C. Cocking, *Analyst*, **80**, 209 (1955).
- 9) K. Morihara, T. Oka and H. Tsuzuki, *Arch. Biochem. Biophys.*, **138**, 515 (1970).
- 10) H. C. Ku, L. Wyborny and G. Kalnitsky, *Biochim. Biophys. Acta*, **268**, 225 (1972).
- 11) I. Schechter and A. Berger, *Biochem. Biophys. Res. Commun.*, **27**, 157 (1967).

TABLE V. EFFECT OF PEPTIDE CHLOROMETHYL KETONE DERIVATIVES FOR INACTIVATION OF PROTEINASE K

Inhibitor	Inactivation ($k \times 10^5$, sec ⁻¹)		
	Proteinase K	Alkaline proteinase ^{a)} from <i>A. sojae</i>	Subtilisin BPN ^{a)}
Cbz Phe CH ₂ Cl	6.7	1.0	1.0
Cbz Ala Phe CH ₂ Cl	36.0	7.0	7.9
Cbz Ala Gly Phe CH ₂ Cl	638.0	90.0	260.0

^{a)} Data from Morihara *et al.*^{4,9)}