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Note

Substrate Specificity of Alkaline Proteases from *Cephalosporium* sp. KM388

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Serine alkaline proteases from *Cephalosporium* sp. KM388 were specific against esters of aromatic and hydrophobic amino acids. Against oxidized insulin B-chain, the enzymes initially cleaved the site of Leu-Tyr(15–16). The cleavage specificity of KM388 protease D was broader than those of other alkaline proteases, and the site of Arg-Gly(22–23) was cleaved, which is a specific site for trypsin-like protease.

Two kinds of serine alkaline proteases (designated KM388 proteases C and D) from *Cephalosporium* sp. KM388 were purified and characterized for our previous paper.[1] The substrate specificity of alkaline proteases from various microbial origins has been investigated with various synthetic substrates and oxidized insulin A- and B-chains. But the substrate specificity of serine alkaline proteases from *Cephalosporium*[2-4] has not been investigated with any natural peptides. Many reports have appeared on the reverse reactions of proteases, *i.e.*, peptide bond synthesis.[5] A protease with a broad substrate specificity is thought to be useful for enzyme-catalyzed peptide synthesis. Therefore, the substrate specificity of serine alkaline proteases of *Cephalosporium* sp. KM388 was investigated using various synthetic substrates and oxidized insulin B-chain.

Activity on amino acid esters was measured by titration with 5 to 10 mM NaOH using a pH-stat apparatus (model AT-117, Kyoto Electronics, Kyoto) at pH 8.0 and 30°C. Substrate was dissolved in 0.1 M KCl containing 10 mM $CaCl_2$ and 10% CH_3CN. The reaction was started by adding the enzyme solution of 0.2 ml to the substrate solution of 7.8 ml, which was incubated for 5 min at 30°C. The amino acid esters used in this work, except for bezoyl arginine ethyl ester (Bz-Arg-OEt), tosyl arginine methyl ester (Tos-Arg-OMe) and Bz-Tyr-OMe, were synthesized by the method of Whitaker.[6] Activities for amino acid amides and peptides were measured by the ninhydrin method[3] after reaction with 0.1 mM of substrate concentration in 10 mM borate buffer (pH 10.0) containing 10 mM $CaCl_2$ and 10% CH_3CN for 20 min at 30°C. KM388 proteases C and D had similar specificity against synthetic amino acid esters as shown in Table. Both KM388 proteases showed the highest activity against Bz-Tyr-OEt, an ester of an aromatic amino acid with a polar side chain. But the enzymes had less activity against Bz-Phe-OEt, an ester of an aromatic amino acid with a nonpolar side chain, than those against Bz-Leu-OMe and Bz-Met-OMe, esters of hydrophobic amino acids with large aliphatic side chains. However, the enzymes hardly hydrolyzed amino acid esters with a substituent on the β-carbon such as Bz-Ile-OMe, Bz-Thr-OMe, and Bz-Val-OMe or a small side chain such as Bz-Ala-OMe, and basic amino acid esters such as Bz-Arg-OEt and Tos-Arg-OMe. These specificity of KM388 proteases for synthetic amino acid esters is similar to that of subtilisin-like proteases.[7,8] However, the amidase activity of the enzymes was not so high. KM388 protease D hydrolyzed Bz-Leu-NH_2 and carbobenzoxy phenylalanyl-tyrosyl-leucine (Z-Phe-Tyr-Leu), although the enzyme did not hydrolyze Bz-Tyr-NH_2 or Z-Phe-Tyr (data not shown). This enzyme also cleaved Z-Gly-Phe, but not Gly-Phe, without modification of the amino group of glycine (data not shown).

For identification of the cleavage site, oxidized insulin B-chain (1 mM) in 10 mM borate buffer (pH 10.0) containing 10 mM $CaCl_2$ was hydrolyzed by KM388 protease C or D (each 1 μM) for 10 min or 1 h at 30°C. The reaction was stopped by freezing the solution with liquid nitrogen. The hydrolyzed peptide fragments were purified by high pressure liquid chromatography and lyophilized. A part of the peptide preparations were hydrolyzed with 6 N HCl

Table Kinetic Parameters for Various Synthetic Amino Acid Esters by Alkaline Proteases of *Cephalosporium* sp. KM388

Amino acid esters	KM388 Protease C			KM388 Protease D		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Bz-Arg-OEt	10.7	23.1	2.2	14.1	54.8	3.9
Tos-Arg-OMe	116.0	78.8	0.7	65.8	84.2	1.3
Bz-Tyr-OEt	10.1	1015.4	100.5	5.7	580.9	101.9
Bz-Phe-OEt	16.6	359.6	21.7	10.5	201.0	19,1
Bz-Met-OMe	11.9	533.8	44.9	3.4	191.9	56.4
Bz-Ala-OMe	20.1	81.4	4.0	66.3	158.6	2.4
Bz-Leu-OMe	3.4	237.6	69.9	7.5	172.7	23.0
Bz-Ile-OMe	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*	n.d.*
Bz-Thr-OMe			0.6**			0.7**
Bz-Val-OMe			0.4**			0.8**

Assays were done as described in the text.

* n.d.: Not detected.

** These values were calculated from the initial rate at the substrate concentration of 0.25 mM.

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Abbreviations: Bz, benzoyl; OMe, methyl ester; OEt, ethyl ester; Tos, tosyl; Z, carbobenzoxy.

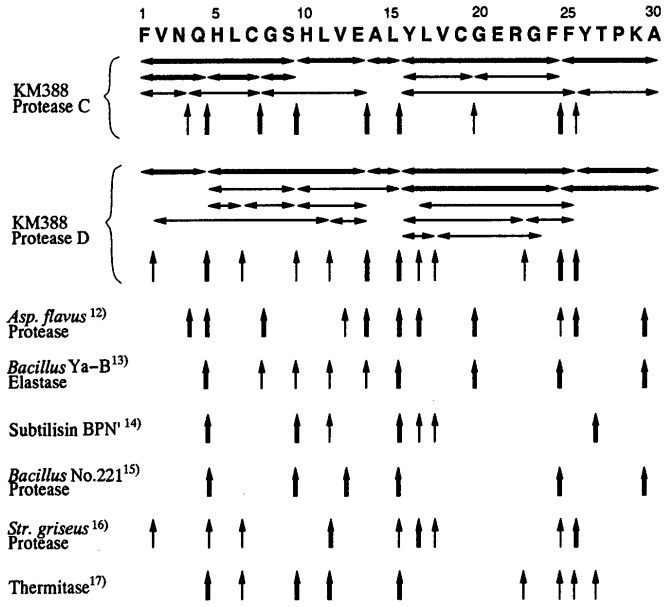

Fig. Cleavage Patterns of Alkaline Proteases from *Cephalosporium* sp. KM388 on Oxidized Insulin B-Chain and Its Comparison with Several Other Alkaline Proteases from Microbial Origin.

Horizontal arrows show cleaved peptide fragments and vertical arrows show cleavage sites. Thick arrows mean major parts and thin arrows mean minor parts.

at 110°C for 24 h and analyzed by an amino acid analyzer (JLC-200A, JOEL, Tokyo). Another part of the peptide preparations was used for the analysis of N-terminal amino acids by the phenyl isothiocyanate method.[9] Phenylthiohidantoin amino acids were identified by thin layer chromatographies on silica gel 60 plates.[10,11] After hydrolysis for 10 min, KM388 protease C gave one major cleavage site at Leu-Tyr(15–16) and four minor cleavage sites at Ser-His(9–10), Glu-Ala(13–14), Phe-Phe(24–25), and Phe-Tyr(25–26). KM388 protease D also showed a similar cleavage pattern. The enzyme, however, gave a minor cleavage site at Gln-His(4–5), but not the site at Ser-His(9–10) (data not shown). These results show that both enzymes initially cleave the site of Leu-Tyr(15–16), which is a common preferable cleavage site among microbial serine alkaline proteases.[8] When oxidized insulin B-chain was hydrolyzed for 1 h, both KM388 proteases showed many cleavage sites as shown in Fig. The cleavage specificity of KM388 protease C resembles those of *Asp. flavus* protease[12] and the alkaline elastase from *Bacillus* sp. Ya-B.[13] On the other hand, the cleavage specificity of KM388 protease D was broader than those of KM388 protease C and other alkaline proteases. It is noteworthy that KM388 protease D also cleaved the site of Arg-Gly(22–23) like thermitase,[17] of which the site is specific to trypsin-like protease.[8]

The broad substrate specificity of alkaline protease D from *Cephalosporium* sp. KM388 resulted in high ability of peptide syntheses based on an aminolysis reaction. This ability of peptide syntheses will be reported elsewhere.

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