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Note

P1-Specificity of Aqualysin I (a Subtilisin-type Serine Protease) from *Thermus aquaticus* YT-1, using P1-Substituted Derivatives of *Streptomyces* Subtilisin Inhibitor

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Aqualysin I is an alkaline serine protease isolated from *Thermus aquaticus* YT-1, an extreme thermophile. We have measured the P1-specificity of aqualysin I, using wild-type and five P1-substituted derivatives of *Streptomyces* subtilisin inhibitor (SSI). SSIs efficiently inhibited the activity of aqualysin I, with low substrate specificity. Charge and hydrophobicity of side chain of the P1 amino acid residue showed no significant effect to the P1-specificity of this enzyme.

Key words: aqualysin I; alkaline serine protease; subtilisin; substrate specificity; *Streptomyces* subtilisin inhibitor

Aqualysin I is an alkaline serine protease isolated from prokaryotic origin *Thermus aquaticus* YT-1, an extreme thermophile.[1–8] The gene coding for this enzyme has been cloned, and its amino acids sequenced. The primary structure of mature protein is similar to those of *Bacillus* subtilisins and fungus proteinase K[5] (the identities in amino acid sequences are around 40%). Aqualysin I has four cysteine residues, like proteinase K, making two disulfide linkages,[4] which are expected to contribute to the thermostability of this protein. This protease displays broad specificity for cleavage of insulin B-chain,[2] and hydrolyzes elastic substrates such as succinyl-$(Ala)_n$-*p*-nitroanilide (n=1, 2, 3) and some peptide esters.[2] Results from these studies indicate that there exist subsites, S1, S2, and S3, within the substrate binding site of aqualysin I, and a tripeptide length is enough for substrate binding and hydrolysis. The substrate specificity of aqualysin I has been analyzed using synthetic tripeptide substrates (Tanaka, T., Matsuzawa,H., and Ohta, T., unpublished results). Results from these experiments showed that the S1 site of aqualysin I prefers alanine and phenylalanine residues to valine and leucine residues. The P1-specificities of acidic and basic amino acid residues have not yet been analyzed. Because of technical difficulties in synthesizing P1-derived chromogenic peptide substrates such as succinyl-X-X-Arg-*p*-nitroanilide and succinyl-X-X-Glu-*p*-nitroanilide, we have decided to use P1-derived protein protease inhibitors for analysis of the P1-specificity of aqualysin I.

Streptomyces subtilisin inhibitor (SSI), a protease inhibitor produced by *Streptomyces albogriseolus*, was isolated, and its primary structure has been identified.[9–13] The protein consisted of 113 amino acid residues. The tertiary structure as well as primary structure has been studied, and the reactive site of the protein has been located.[14] Met-73 in SSI is known as the reactive site of subtilisin, as the P1 amino acid residue, many P1-substituted derivatives of SSI were prepared by site-directed mutagenesis.[12–13]

In this paper, we have analyzed the P1-specificity of aqualysin I, using wild-type and five P1-substituted derivatives of SSI as substrate analogues. And we have also analyzed P1-specificities of microbial alkaline serine proteases, proteinase K, subtilisin Carlsberg, and subtilisin BPN', under the standard conditions for aqualysin I.

Aqualysin I was purified from the culture medium of *Thermus aquaticus* YT-1 by the method described previously.[2] Proteinase K (Merck, lot 710 E609668), subtilisin Carlsberg (Sigma Chemical Co., No. P-5380, lot 18F-0005), and subtilisin BPN' (Nagase Biochemicals. Ltd., Bacterial A1-Proteinase Nagarse, lot 6928013) were purchased, and were further purified to homogeneity with column chromatography using a FPLC system with a mono-S column, because commercially available proteases are not always pure, containing degraded products. The protein concentration of each enzyme

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Abbreviations: HEPES, *N*-(2-Hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; FPLC, Fast Protein Liquid Chromatography; ε_{410}, Molecular extinction coefficient at 410 nm; k_{cat}, Catalytic rate constant; K_m, Michaelis constant; $K_{i(int)}$, intrinsic inhibition constant; SSI, *Streptomyces* subtilisin inhibitor.

was measured by a BCA Protein Assay kit (Pierce). Wild-type and P1-substituted SSIs, replacing Met-73 by alanine, phenylalanine, leucine, aspartic acid, or arginine, were purified by the method described previously.[11-13]

The chromogenic tetrapeptide substrate succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide was purchased from Sigma Chemical Co. Succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide is often used as a standard substrate for subtilisin BPN',[13,16-17] and is efficiently hydrolyzed by subtilisin BPN' and subtilisin Carlsberg. Succinyl-Phe-Nle-Ala-*p*-nitroanilide was synthesized step by step in the liquid phase using the mixed anhydride method from *p*-nitroanilide derivatives of amino acids, adding *tert*-butoxycarbonyl acylated amino acids to elongate toward the amino-terminus through reactions. All derivatives of amino acids for synthesis were purchased from Kokusan Chemical Works Ltd. Aqualysin I prefers this substrate, and hydrolyzes it with high efficiency. This tripeptide substrate is also hydrolyzed efficiently by proteinase K, subtilisin BPN', and subtilisin Carlsberg (Tanaka, T., Matsuzawa, H., and Ohta, T., unpublished results). So, succinyl-Phe-Nle-Ala-*p*-nitroanilide was used for the assays of aqualysin I and proteinase K as a standard substrate, and succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide was used for subtilisin BPN' and subtilisin Carlsberg.

Each substrate was dissolved in HEPES buffer (100 mM HEPES, 1 mM $CaCl_2$, pH 7.5 at 40°C) over the solubility limit of the reagent before use, and the solution was passed through a filter (0.22-μm pore size) to remove the undissolved excess. The substrate concentration was measured spectrophotometrically from the absorbance of released *p*-nitroaniline ($\varepsilon_{410}=8680\ cm^{-1}\ M^{-1}$) after complete hydrolysis. Reactions were started by addition of enzyme solution (10–50 nM, 30 μl) to substrate solution (270 μl) in a quartz cell on a spectrophotometer equipped with a thermostatted cell compartment, then the release of *p*-nitroaniline was monitored at 410 nm. Spontaneous hydrolysis of two peptide substrates were small enough within experimental error. Kinetic parameters, k_{cat} and K_m, were measured from the initial rate measurements for hydrolysis of *p*-nitroanilide substrates. Data were fit to the Michaelis-Menten equation using a nonlinear regression algorithm.

Kinetic parameters for the hydrolysis of two chromogenic substrates are shown in Table 1. All enzymes hydrolyzed both peptide substrates efficiently. Hydrolysis of succinyl-Phe-Nle-Ala-*p*-nitroanilide was accompanied with a small K_m, while hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide was accompanied by a large k_{cat}.

Inhibition assays were done by the methods described previously.[13,15] Each SSI-derivative were mixed first with each enzyme at various concentrations in HEPES-buffer, incubated for 30 min at 40°C, and measurement of the residual activities were followed. The apparent inhibition constant, $K_{i(app)}$, was calculated from the equation as follows;

$$\alpha(=[E]/[E_0])=(([E_0]-[I_0]-K_{i(app)}) + (([E_0]-[I_0]-K_{i(app)})^2+4[E_0]K_{i(app)})^{0.5}) /2[E_0],$$

where [E] means free enzyme concentration, $[E_0]$ means total enzyme concentration, and $[I_0]$ means total inhibitor concentration. The intrinsic inhibition constant, $K_{i(int)}$, was calculated from the apparent inhibition constant, $K_{i(app)}$, according to the equation:

$$K_{i(int)}=K_{i(app)}/(1+[S]/K_m).$$

The inhibition constants of wild-type and P1-substituted derivatives of SSI toward proteases are shown in Table 2. P1-substituted SSIs as well as wild-type SSI bound to aqualysin I and inhibited the activity of this enzyme efficiently. The magnitude of inhibition constants

Table 2. Inhibition Constants of P1-Substituted Derivatives of SSI toward Proteases

P1	Aqualysin I	Proteinase K	Subtilisin Carlsberg	Subtilisin BPN'
Met*	3.0×10^{-10}	1.5×10^{-9}	7.6×10^{-11}	9.6×10^{-11}
Ala	3.8×10^{-10}	2.2×10^{-9}	4.2×10^{-12}	6.0×10^{-11}
Phe	3.6×10^{-10}	1.8×10^{-9}	6.4×10^{-11}	7.2×10^{-11}
Leu	5.9×10^{-10}	2.6×10^{-9}	1.9×10^{-11}	8.2×10^{-11}
Asp	1.7×10^{-10}	1.0×10^{-8}	5.0×10^{-9}	4.0×10^{-9}
Arg	8.1×10^{-11}	1.1×10^{-9}	1.3×10^{-10}	6.6×10^{-11}

* Corresponding to the wild-type SSI. Tripeptide substrate succinyl-Phe-Nle-Ala-*p*-nitroanilide (about 50 μM) was used for the assays of aqualysin I and proteinase K, and tetrapeptide substrate succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (about 0.5 mM) was used for the assays of subtilisin BPN' and subtilisin Carlsberg. Assays were done at 40°C, pH 7.5 (100 mM HEPES, 1 mM $CaCl_2$). 100 μl of enzyme solution (about 10–50 nM) and 100 μl of various concentrations of SSI solution were mixed and were incubated for 30 min at 40°C. After incubation of the mixture, residual activity of the enzyme was measured by the addition of 200 μl of pre-incubated (at 40°C) substrate solution. The intrinsic inhibition constant $K_{i(int)}$ was calculated from the apparent inhibition constant $K_{i(app)}$, using the value of the Michaelis constant shown in Table 1.

Table 1. Kinetic Parameters of Proteases for the Hydrolysis of Succinyl-peptide-*p*-nitroanilides

Enzyme	suc-Phe-Nle-Ala-pNA			suc-Ala-Ala-Pro-Phe-pNA		
	k_{cat} [sec^{-1}]	K_m [M]	k_{cat}/K_m [$sec^{-1}\ M^{-1}$]	k_{cat} [sec^{-1}]	K_m [M]	k_{cat}/K_m [$sec^{-1}\ M^{-1}$]
Aqualysin I	8.8	3.6×10^{-5}	2.4×10^{5}	3.3×10^{1}	1.2×10^{-3}	2.7×10^{4}
Proteinase K	7.9	6.0×10^{-5}	1.3×10^{5}	1.1×10^{4}	3.4×10^{-4}	3.2×10^{7}
Subtilisin BPN'	2.8×10^{1}	2.4×10^{-5}	1.2×10^{6}	4.8×10^{2}	2.9×10^{-4}	1.7×10^{6}
Subtilisin Carlsberg	3.3	5.0×10^{-5}	6.6×10^{5}	3.0×10^{2}	2.9×10^{-4}	1.0×10^{6}

Assays were done at 40°C, pH 7.5 (100 mM HEPES, 1 mM $CaCl_2$).

of P1-substituted and wild-type SSI toward aqualysin I were around 10^{-10} M. The inhibition constant of SSI containing hydrophobic residues such as alanine and phenylalanine in the position of the P1 site were almost of the same magnitude as that of methionine-containing SSI. The inhibition of Leu-73 SSI (P1-site changed leucine) was weaker than that of wild-type SSI. So, the S1 site of aqualysin I preferred alanine and phenylalanine as well as methione to leucine. The inhibition constant of Leu-73 SSI was only two times that of Ala-73 SSI, while the Michaelis constant of the leucine-containing substrate in P1 site (succinyl-Phe-Val-Leu-*p*-nitroanilide) was about ten times that of the alanine-containing substrate (succinyl-Phe-Val-Ala-*p*-nitroanilide) (Tanaka, T., Matsuzawa, H., and Ohta, T., unpublished results). Inhibition constants of proteinase K and subtilisin BPN' displayed the same tendencies. The S1 sites of these proteases also preferred alanine and phenylalanine to leucine residues, while no significant differences in the magnitude of inhibition constants were detected. However, the results for subtilisin Carlsberg differed from the others. Subtilisin Carlsberg also preferred alanine to leucine residue, and the P1-specificity with peptide substrates is consistent with the results of the inhibition constants. The inhibition constant of Leu-73 SSI was about four times that of Ala-73 SSI.

SSI, containing aspartic acid and arginine, also bound tightly to aqualysin I and inhibited the activity of this enzyme. The inhibition constant of Asp-73 SSI was about half of that of wild-type SSI, and the inhibition constant of Arg-73 SSI was about a quarter of that of wild-type SSI. The S1 site of aqualysin I preferred both acidic aspartate and basic arginine to hydrophobic residues such as alanine, phenylalanine, and leucine. Asp-73 SSI also bound to proteinase K, subtilisin BPN', and subtilisin Carlsberg. The inhibition constant of Asp-73 SSI toward proteinase K was about seven times that of wild-type SSI, and that toward subtilisin Carlsberg was about 66 times that of wild-type SSI, and also that toward subtilisin BPN' was about 42 times that of wild-type SSI. The S1 sites of proteinase K, subtilisin BPN', and subtilisin Carlsberg did not prefer aspartic acid residues. The results of subtilisin BPN' were consistent with the results previously reported.[14-15] Electrostatic interactions between Glu-156 within the S1 site of subtilisin BPN' and the side chain of the P1 site amino acid residue of the substrate contributed to the substrate specificity. However, this explains the recognition mechanism only in the case of subtilisin BPN'. The corresponding amino acid residue of other enzymes differ; Ser-156 of subtilisin Carlsberg, and Asn-162 of proteinase K, not acidic residues. These results suggest that Glu-156 of subtilisin BPN' does not always have an important role in the recognition of the P1 amino acid residue. The corresponding amino acid of aqualysin I is Asp-158, an acidic residue, and the electrostatic interactions of Asp-158 with Asp-73 of SSI was expected to be weak. The inhibition constant of Arg-73 SSI toward proteinase K and subtilisin BPN' were almost the same magnitude as wild-type SSI. The inhibition constant of Arg-73 SSI toward subtilisin Carlsberg was about two times that of wild-type SSI. Positive electrostatic interactions between the S1 site of subtilisin Carlsberg and arginine of SSI was not observed. The inhibition constants measured in this study were consistent with the previous data,[13] except that the values were slightly larger. These differences may be due to the differences of assay conditions. Previous experiments were done under the standard conditions for subtilisin BPN' (pH 8.6, 25°C; 100 mM Tris-HCl), not under the standard conditions for aqualysin I.

Compared with proteinase K and the two subtilisins, aqualysin I has low P1-specificity. These data suggest that the recognition mechanism of P1-site of aqualysin I is different from those of proteinase K and the two subtilisins, or there is something around the substrate binding site of aqualysin I that prevents efficient recognition of P1 site residues.

References

1) Matsuzawa, H., Hamaoki, M., and Ohta, T., Production of thermophilic extracellular proteases (aqualysins I and II) by *Thermus aquaticus* YT-1, an extreme thermophile. *Agric. Biol. Chem.*, **47**, 25–28 (1983).
2) Matsuzawa, H., Tokugawa, K., Hamaoki, M., Mizoguchi, M., Taguchi, H., Terada, I., Kwon, S.-T., and Ohta, T., Purification and characterization of aqualysin I (a thermophilic alkaline serine protease) produced by *Thermus aquaticus* YT-1. *Eur. J. Biochem.*, **171**, 441–447 (1988).
3) Kwon, S.-T., Terada, I., Matsuzawa, H., and Ohta, T., Nucleotide sequence of the gene for aqualysin I (a thermophilic alkaline serine protease) of *Thermus aquaticus* YT-1 and characteristics of the deduced primary structure of the enzyme. *Eur. J. Biochem.*, **173**, 491–497 (1988).
4) Kwon, S.-T., Matsuzawa, H., and Ohta, T., Determination of the positions of the disulfide bonds in aqualysin I (a thermophilic alkaline serine protease) of *Thermus aquaticus* YT-1. *J. Biochem.*, **104**, 557–559 (1988).
5) Terada, I., Kwon, S.-T., Miyata, Y., Matsuzawa, H., and Ohta, T., Unique precursor structure of an extracellular protease, aqualysin I, with NH_2- and COOH-terminal pro-sequences and its processing in *Escherichia coli*. *J. Biol. Chem.*, **265**, 6576–6581 (1990).
6) Lee, Y.-C., Miyata, Y., Terada, I., Ohta, T., and Matsuzawa, H., Involvement of NH_2-terminal pro-sequence in the position of active aqualysin I (a thermophilic serine protease) in *Escherichia coli*. *Agric. Biol. Chem.*, **55**, 3027–3032 (1991).
7) Lee, Y.-C., Ohta, T., and Matsuzawa, H., A non-covalent NH_2-terminal pro-region aids the production of active aqualysin I (a thermophilic protease) without the COOH-terminal pro-sequence in *Escherichia coli*. *FEMS Microbiol. Lett.*, **92**, 73–78 (1992).
8) Tanaka, T., Matsuzawa, H., and Ohta, T., Stability of thermostable enzyme, aqualysin I; a subtilisin-type serine protease from *Thermus aquaticus* YT-1. *Biosci. Biotechnol. Biochem.*, in press.
9) Ikenaka, T., Odani, S., Sakai, M., Nabeshima, Y., and Murao, S., Amino acid sequence of an alkaline proteinase inhibitor (*Streptomyces* subtilisin inhibitor) from *Streptomyces albogriseolus* S-3253. *J. Biochem.*, **76**, 1191–1209 (1974).
10) Obata, S., Taguchi, S., Kumagai, S., and Miura, K., Molecular cloning and nucleotide sequence determination of gene encoding *Streptomyces* subtilisin inhibitor (SSI). *J. Biochem.*, **105**, 367–371 (1989).
11) Obata, S., Furukubo, S., Kumagai, I., Takahashi, H., and Miura, K., High-level expression in *Streptomyces lividans* 66 of a gene encoding *Streptomyces* subtilisin inhibitor from *Streptomyces albogriseolus* S-3253. *J. Biochem.*, **105**, 372–376 (1989).
12) Miura, K., Kumagai, I., Obata, S., Kojima, S., and Taguchi, S.,

Partial alternation of a protein *Streptomyces* subtilisin inhibitor by site-directed mutagenesis. *Proc. Japan Acad.*, **64**, 147–149 (1988).

13) Kojima, S., Nishiyama, Y., Kumagai, I., and Miura, K., Inhibition of subtilisin BPN' by reaction site P1 mutants of Streptomyces subtilisin inhibitor. *J. Biochem.*, **109**, 377–382.

14) Ishikawa, K., Mitsui, Y., and Iitaka, Y., Aspects of protein-protein interaction as studied through crystal structure of subtilisin complexed with its trapped substrate SSI. *Proc. Symposium on Three-dimensional Structure and Drug Action.*, 165–175 (1987).

15) Hiromi, K., Akasaka, K., Mitsui, Y., Tonomura, B., and Murao, S., eds., *Protein Protease Inhibitor—The Case of Streptomyces Subtilisin Inhibitor* (*SSI*). Elsevier Science Publishers B.V., Amsterdam (1985).

16) Wells, J. A., Powers, D. B., Bott, R. R., Graycar, T. P., and Estell, D. A., Designing substrate specificity by protein engineering of electrostatic interactions. *Proc. Natl. Acad. Sci., USA*, **84**, 1219–1223 (1987).

17) Russell, A. J. and Fersht, A. R., Rational modification of enzyme catalysis by engineering surface charge. *Nature*, **328**, 496–500 (1987).