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Note

Substrate Specificity of Aqualysin I Altered by an Organic Solvent, DMSO

Terumichi TANAKA,† Hiroshi MATSUZAWA, and Takahisa OHTA*

Department of Biotechnology, The University of Tokyo, Bunkyo-ku, Tokyo 113-8657, Japan

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Aqualysin I is the alkaline serine protease isolated from an extreme thermophile, *Thermus aquaticus* YT-1. We have analyzed the kinetic properties of aqualysin I, using thirty-one kinds of chromogenic succinyl-tripeptide *p*-nitroanilides as substrates in the presence of 10% dimethylsulfoxide (DMSO). Aqualysin I hydrolyzed many peptides in a DMSO-containing mixture, however the substrate specificity was different from that in the absence of DMSO. The K_m for each peptide was raised by the addition of 10% DMSO. Also, the P3- as well as P2-specificity of aqualysin I was altered. These results suggested that the side chains of the P2 and P3 residues are exposed to the solvent, and the hydrophobic interactions between the side chain of the substrate and the solvent may take a part in the substrate recognition of the enzyme.

Key words: aqualysin I; alkaline serine protease; DMSO; substrate specificity; *Thermus aquaticus* YT-1

Aqualysin I is the alkaline serine protease isolated from the prokaryote *Thermus aquaticus* YT-1, an extreme thermophile.[1-11] The gene encoding this enzyme was cloned, and its amino acids sequenced. The primary structure of the mature protein is similar to those of *Bacillus* subtilisins and fungus proteinase K (the identities are around 40%).[2] Aqualysin I has four cystein residues, like proteinase K, making two disulfide linkages,[4] which are expected to contribute to the stability of this thermostable protein.[2,8] This protease has a broad specificity for cleavage of insulin B-chain,[2] and hydrolyzes synthetic chromogenic substrates such as suc-tripeptide-pNA.[10] Results from these studies indicated that aqualysin I has subsites, S1, S2, and S3, within the substrate binding site. However, we have little information about the tertiary structure of aqualysin I. Comparative kinetic studies of aqualysin I with subtilisin BPN′, subtilisin Carlsberg, and proteinase K under the same conditions,[9,10] and moreover, the results of the alteration of the P2-specificity of aqualysin I done on the basis of the structural similarity,[11] suggested that the structure of the substrate binding site of aqualysin I may be identical with those of subtilisins as predicted by other report on subtilisins earlier.[12]

Crystallographic structures of subtilisins showed that the S2 site forms a half-sphere shaped pocket and the S3 site forms a cleft.[12-17] No bulky amino acid residues exist around the S3 site, and the side chain of the P3 amino acid residue is exposed to the solvent molecules and is directed toward the outside of the enzyme. These results suggested that the hydrophobicity of the solvent may affect the P2- and P3-specificity of the enzyme, and also that the organic solvent can be used as a probe to examine not only the interactions between the side chains of the substrate and the enzyme surface but also the interactions between the side chain of the substrate and the solvent.

In this paper, we have measured the P2- and P3-specificities of aqualysin I, using thirty one chromogenic peptides, to examine the substrate specificity of this enzyme in the presence of 10% DMSO, and also the effects of DMSO on substrate specificity.

As previous studies on the substrate specificity of aqualysin I in the absence of DMSO showed that the S1 site of this enzyme prefers alanine,[10] we decided to use peptides as substrates the P1 site of which are fixed as alanine. Alanine-containing peptides in the P1 site are easy to synthesize and are soluble in hydrous solution. Thirty-one tripeptides, containing alanine in P1 site, were prepared to examine the substrate specificity of aqualysin I in the presence of DMSO. Twenty-one polypeptides were cleaved by aqualysin I. Kinetic parameters are summarized in Table 1.

The hydrolyses of suc-Ile-Gly-Ala-pNA, suc-Gly-Val-Ala-pNA, suc-Val-Leu-Ala-pNA, suc-Leu-Ile-Ala-pNA, suc-X-Pro-Ala-pNA (X=Ala, or Ile), and suc-X-βAla-Ala-pNA (X=Gly, Phe, Leu, or Ile) were not detected, even in the presence of 10 μM concentration of the enzyme. β-Alanine-containing peptides were not recognized as substrates both in the presence and in the absence of DMSO. Peptides containing glycine or proline in the P2 site, or peptides containing glycine in the P3 site were not good substrates for aqualysin I under this condition. Efficient hydrolyses were observed with the substrate suc-X-Val-Ala-pNA (X=Phe, Ile, Val, Pro, or Tyr). These results showed that the S2 site of aqualysin I preferred valine to the other residues, glycine, alanine, leucine, isoleucine, and proline. Compared to the P2-specificity, the P3-specificity of this enzyme under this condition was wobbly. Comparison of

† To whom correspondence should be addressed.
† Present address: *Division of Bioscience and Biotechnology, Department of Ecological Engineering, Toyohashi University of Technology, Tempaku-cho, Toyohashi, Aichi 441-8580, Japan.* Tel : +81-532-44-6912; Fax: +81-532-44-6929; E-mail: tanakat@eco.tut.ac.jp
* Present address: *Kogakuin University, 1-24-2, Nishi-shinjuku, Shinjuku-ku, Tokyo, 163, Japan*

Abbreviations: HEPES, N-(2-Hydroxyethyl)piperazine-N′-2-ethanesulfonic acid; ε_{410}, Molecular extinction coefficient at 410 nm; DMSO, Dimethylsulfoxide; k_{cat}, Catalytic rate constant; K_m, Michaelis constant; Suc-, Succinyl-(3-Carboxypropyl-); -pNA, *p*-nitroanilide

Table 1. Substrate Specificity of Aqualysin I in the Presence of 10% DMSO

P3	P2	Containing 10% DMSO[a] k_{cat} [s^{-1}]	K_m [M]	k_{cat}/K_m [$M^{-1}s^{-1}$]	Without DMSO[b] k_{cat} [s^{-1}]	K_m [M]	k_{cat}/K_m [$M^{-1}s^{-1}$]
Gly	Gly	—	—	3.1			
Leu	Gly	2.9×10^{-1}	2.3×10^{-2}	1.3×10^{1}			
Ile	Gly	—	—	ND			
Phe	Gly	3.2×10^{-1}	3.0×10^{-3}	1.0×10^{2}			
Ala	Ala	1.1	9.3×10^{-3}	1.2×10^{2}	1.4	1.3×10^{-3}	1.1×10^{3}
Val	Ala	6.2×10^{-1}	4.1×10^{-3}	1.5×10^{2}			
Leu	Ala	7.5×10^{-1}	5.3×10^{-3}	1.4×10^{2}			
Ile	Ala	3.0×10^{-1}	1.6×10^{-3}	1.8×10^{2}			
Phe	Ala	1.8×10^{-1}	2.2×10^{-3}	8.0×10^{1}	1.0×10^{1}	4.0×10^{-5}	2.6×10^{5}
Gly	Val	—	—	ND			
Ala	Val	1.8	1.6×10^{-2}	1.2×10^{2}			
Val	Val	4.0	4.3×10^{-3}	9.4×10^{2}			
Leu	Val	—	—	1.3×10^{2}			
Ile	Val	8.1	1.0×10^{-3}	7.7×10^{3}	5.0	6.4×10^{-5}	7.8×10^{4}
Phe	Val	9.1	4.4×10^{-4}	2.1×10^{4}	4.7	3.9×10^{-5}	1.2×10^{5}
Tyr	Val	4.3×10^{1}	3.6×10^{-2}	1.2×10^{3}			
Pro	Val	5.7	6.4×10^{-3}	8.9×10^{2}			
Ala	Leu	3.1×10^{-1}	7.2×10^{-3}	4.3×10^{1}			
Val	Leu	—	—	ND			
Phe	Leu	4.3×10^{-1}	3.1×10^{-3}	1.4×10^{2}	3.6	8.7×10^{-5}	4.1×10^{4}
Ala	Ile	1.1	1.1×10^{-2}	9.3×10^{1}			
Leu	Ile	—	—	ND			
Gly	Pro	—	—	1.7×10^{1}			
Ala	Pro	—	—	ND	4.8×10^{-1}	7.9×10^{-4}	6.1×10^{2}
Leu	Pro	3.7×10^{-1}	1.3×10^{-2}	2.9×10^{1}			
Ile	Pro	—	—	ND			
Phe	Pro	2.0×10^{-1}	5.3×10^{-3}	3.9×10^{1}			
Gly	βAla	—	—	ND	—	—	ND
Phe	βAla	—	—	ND	—	—	ND
Leu	βAla	—	—	ND	—	—	ND
Ile	βAla	—	—	ND	—	—	ND

Kinetic parameters for the hydrolysis of suc-P3-P2-Ala-pNA with 10% DMSO in the reaction mixture. Assays were done at 40°C, pH 7.5 (100 mM HEPES, 1 mM $CaCl_2$, 10% DMSO). Aqualysin I was purified from the culture medium of *Thermus aquaticus* YT-1 according to the method described previously.[2] All chromogenic suc-tripeptide-pNAs were kindly supplied by Dr. K. Asano, Kirin Brewery Co., Ltd, Pharmaceutical Laboratory. Reactions were started by addition of enzyme solution (30 μl) to substrate solution (270 μl; containing 12.5% DMSO) in a quartz cell in a spectrophotometer with a thermostatted cell compartment, then the release of *p*-nitroaniline was monitored at 410 nm. Kinetic parameters were calculated from initial rate measurements for hydrolysis of *p*-nitroanilide substrate, by fitting to the Michaelis-Menten equation using a nonlinear regression algorithm as described earlier.[10] The enzyme concentrations used for the hydrolyses of peptides were around 10 nM-10 μM. Standard errors were less than 35%. [a]This work.[b] The kinetic parameters under the standard assay conditions for aqualysin I. The assays were done in the absence of DMSO (100 mM HEPES, 1 mM $CaCl_2$, at 40°C, pH 7.5). Data are from the previous work.[10] The values of k_{cat} and K_m for suc-P3-P2-Ala-pNA (P3-P2=Gly-Gly, Leu-Val, Gly-Pro) were not calculated because of the large value of K_m over the solubility limit of the substrate, therefore only the value of apparent second-order rate constant, k_{cat}/K_m, was measured. "ND" stands for "the hydrolysis of the peptide was not detected".

kinetic parameters for suc-X-Val-Ala-pNA (X=Phe, Ile, Val, Pro, or Tyr) showed that the S3 site of aqualysin I preferred bulky residues, phenylalanine, tyrosine, and isoleucine, to other residues. However, comparison of kinetic parameters for suc-X-Ala-Ala-pNA (X=Phe, or Ala) showed that the S3 site of this enzyme preferred alanine to phenylalanine. The P3-specificity of aqualysin I was affected by the kind of the P2 residue of the peptide substrate.

Table 2. Comparison of P2- and P3-Specificity in the Presence/Absence of DMSO

[A] Comparison of parameters for suc-Phe-X-Ala-pNA (X=Val, Leu, Ala)

P2 residue	DMSO[a] k_{cat}	DMSO[a] K_m	Reference[b] k_{cat}	Reference[b] K_m	Ratio $k_{cat}^{DMSO}/k_{cat}^{ref}$	Ratio K_m^{DMSO}/K_m^{ref}
Ala	0.18	2.2	10	0.040	0.018	55
Val	9.1	0.44	4.7	0.039	1.9	11
Leu	0.43	3.1	3.6	0.087	0.12	36
Ratio (Val/Ala)	51	20	0.47	0.98		
Ratio (Leu/Ala)	2.4	1.4	0.36	2.2		
Ratio (Val/Leu)	21	0.14	1.3	0.45		

[B] Comparison of parameters for suc-X-Val-Ala-pNA (X=Phe, Ile)

P3 residue	DMSO[a] k_{cat}	DMSO[a] K_m	Reference[b] k_{cat}	Reference[b] K_m	Ratio $k_{cat}^{DMSO}/k_{cat}^{ref}$	Ratio K_m^{DMSO}/K_m^{ref}
Ile	8.1	1.0	5.0	0.064	1.6	16
Phe	9.1	4.4	4.7	0.039	1.9	11
Ratio (Phe/Ile)	1.1	0.44	0.94	0.61		

[C] Comparison of parameters for suc-X-Ala-Ala-pNA (X=Phe, Ala)

P3 residue	DMSO[a] k_{cat}	DMSO[a] K_m	Reference[b] k_{cat}	Reference[b] K_m	Ratio $k_{cat}^{DMSO}/k_{cat}^{ref}$	Ratio K_m^{DMSO}/K_m^{ref}
Ala	1.1	9.3	1.4	1.3	0.79	7.2
Phe	0.18	2.2	10	0.04	0.018	55
Ratio (Phe/Ala)	0.16	0.23	7.1	0.031		

Data were from Table 1. Kinetic parameters in the presence of 10% DMSO ([a]) and in the absence of DMSO ([b]), respectively. Units are as follows: k_{cat}, s^{-1}; and K_m, mM.

The kinetic parameters in the presence of DMSO for six peptides (P3-P2=Ala-Ala, Phe-Ala, Ile-Val, Phe-Val, Phe-Leu, or Ala-Pro) were available to compare with those in the absence of DMSO (Table 2). The comparison showed that the K_m of aqualysin I for each peptide was raised by the addition of DMSO. The P2- as well as P3-specificity in the presence of DMSO were different from those in the absence of organic solvent. That is discussed below.

To study the effects of addition of DMSO on the P2-specificity of aqualysin I, the parameters for suc-Phe-X-Ala-pNA (X=Ala, Val, or Leu) in the presence and absence of DMSO were compared (Table 2A). The S2 site of aqualysin I in the absence of organic solvent prefers alanine to valine and leucine, and this site can not distinguish between valine and leucine under this condition. The k_{cat} for suc-Phe-Ala-Ala-pNA in the absence of DMSO is about two or three times of those for suc-Phe-Val-Ala-pNA and suc-Phe-Leu-Ala-pNA, respectively. On the other hand, the S2 site of this enzyme preferred valine to alanine and leucine in the presence of DMSO. The k_{cat} value for suc-Phe-Ala-Ala-pNA was reduced to 1.8% and the k_{cat} value for suc-Phe-Leu-Ala-pNA was

reduced to 12% by addition of 10% DMSO. The S2 site distinguished between valine and leucine under this condition. The P2-specificity of aqualysin I was altered by DMSO.

To analyze the effects of addition of DMSO on the P3-specificity of aqualysin I, the parameters for suc-X-Val-Ala-pNA (X=Phe, or Ile) in the presence/absence of DMSO, and also parameters for suc-X-Ala-Ala-pNA (X=Phe, or Ala) in the presence/absence of DMSO were compared. Comparison of parameters for suc-Ile-Val-Ala-pNA and suc-Phe-Val-Ala-pNA showed that aqualysin I was activated about two times for the hydrolyses of these peptides by the addition of DMSO (Table 2B). The activation of enzyme for the hydrolyses of these peptides were contributed to the valine residue in the P2 site of the substrate. Aqualysin I did not distinguish between isoleucine and phenylalanine in the presence of DMSO as well as in the absence of DMSO. In this case, the P3-specificity of aqualysin I was not affected. However, the results from the studies for suc-X-Ala-Ala-pNA (X=Ala, Phe) were different (Table 2C). Aqualysin I was not activated for the hydrolysis of these peptides. The k_{cat} for suc-Ala-Ala-Ala-pNA in the presence of DMSO was identical with that in the absence of DMSO, however the k_{cat} of suc-Phe-Ala-Ala-pNA was greatly reduced by the addition of DMSO. As a result, the S3 site of aqualysin I was altered to be alanine-preferable in the presence of DMSO, while this subsite prefers phenylalanine in the absence of organic solvent.[10] The P3-specificity of aqualysin I was also altered by the addition of DMSO.

As these results are concerned with the P2- and P3-specificity of aqualysin I, not with the P1-specificity, we cannot predict the effects of organic solvent on the P1-specificity of this enzyme. The results indicated that the K_m of aqualysin I for tripeptide substrates were raised by addition of 10% DMSO, and the P2- as well as P3-specificity of this enzyme were affected. The results suggested that we can alter the substrate specificity of aqualysin I by manipulating the DMSO concentration of the reaction mixture. These results also suggested that the side chains of the P2 and P3 residues are exposed to the solvent, and the hydrophobic interactions between the side chain of the substrate and the solvent may take a part in the substrate recognition of the enzyme.

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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.*, **62**, 1806–1808 (1998).
9) Tanaka, T., Matsuzawa, H., Kojima, S., Kumagai, I., Miura, K., and Ohta, T., P1-specificity of aqualysin I (a subtilisin-type serine protease) from *Thermus aquaticus* YT-1, using P1-substituted derivatives of *Streptomyces* subtilisin inhibitor. *Biosci. Biotechnol. Biochem.*, **62**, 2035–2038 (1998).
10) Tanaka, T., Matsuzawa, H., and Ohta, T., Substrate specificity of aqualysin I, a bacterial thermophilic alkaline serine protease from *Thermus aquaticus* YT-1: comparison with proteinase K, subtilisin BPN′ and subtilisin Carlsberg. *Biosci. Biotechnol. Biochem.*, **62**, 2161–2165 (1998).
11) Tanaka, T., Matsuzawa, H., and Ohta, T., Engineering of S2 site of aqualysin I; alteration of P2-specificity by excluding P2 side chain. *Biochemistry*, **37**, 17402–17407 (1998).
12) Siezen, R. J, Vos, W. M., Leunissen, J. A. M., and Dijkstra, B. W., Homology modelling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. *Protein Eng.*, **4**, 719–737 (1991).
13) Wright, C. S., Alden, R. A., and Kraut, J., Crystal structure of a subtilisin BPN′ complex with N-benzoyl-L-arginine. *J. Mol. Biol.*, **66**, 283–289 (1972).
14) Robertus, J. D., Alden, R. A., Birktoft, J. J., Kraut, J., Powers, J. C., and Wilcox, P. E., An x-ray crystallographic study of the binding peptide chloromethyl ketone inhibitors to subtilisin BPN′. *Biochemistry*, **11**, 2439–2449 (1972).
15) Matthews, D. A., Alden, R. A., Birktoft, J. J., Freer, S. T., and Kraut, J., X-ray crystallographic study of boronic acid adducts with subtilisin BPN′ (Novo). *J. Biol. Chem.*, **250**, 7120–7126 (1975).
16) Bode, W., Papamokos, E., and Musil, D., The high-resolution x-ray crystal structure of the complex formed between subtilisin Carlsberg and eglin c, an elastase inhibitor from the leech *Hirudo medicinalis*. *Eur. J. Biochem.*, **166**, 673–692 (1987).
17) Betzel, C., Pal, G. P., and Saenger, W., Three-dimensional structure of proteinase K at 0.15-nm resolution. *Eur. J. Biochem.*, **178**, 155–171 (1988).