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

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Aqualysin I is the alkaline serine protease isolated from an extreme thermophile, *Thermus aquaticus* YT-1. We analyzed kinetic properties of aqualysin I, using sixteen kinds of chromogenic succinyl-tripeptide p-nitroanilides as substrates. And we compared the substrate specificity of aqualysin I with those of proteinase K, subtilisin BPN’, and subtilisin Carlsberg. We found that aqualysin I had three substrates, S1, S2, and S3, in the substrate binding site. S1 site preferred alanine and phenylalanine. S2 site preferred alanine and norleucine. And S3 site preferred phenylalanine and isoleucine. These specificities were similar to those of protease K and subtilisin BPN’. The specificity of subtilisin Carlsberg differed from those of other enzymes.

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

Aqualysin I is the alkaline serine protease isolated from the prokaryote *Thermus aquaticus* YT-1, an extreme thermophile.1-7,11 The gene encoding this enzyme was cloned, and its amino acid sequence determined. The primary structure of the mature protein is similar to those of *Bacillus* subtilis subtilisin K83 (the homologous identities are around 40%). Aqualysin I has four cysteine residues, like proteinase K, making two disulfide linkages,4,9 which are expected to contribute to the thermostability of aqualysin I. This protease displays broad specificity for cleavage of insulin B-chain,9 and hydrolyzes elastic substrates such as succinyl-(Ala)₃-p-nitroanilide (n = 1, 2, 3) and some peptide esters.9 Results from these studies suggest that there exist substrates, S1, S2, and S3, within the substrate binding site of aqualysin I.

The tertiary structures of proteinase K and some subtilisins are well-defined.4,12 These studies revealed that the substrate binding site of these enzymes includes several substrates, including S1, S2 and S3 sites. Some kinetic analyses supported these data.13,16

In this paper, we determined kinetic parameters of aqualysin I, using chromogenic succinyl-peptide p-nitroanilides, to confirm the existence of the substrates S1, S2, and S3, and to obtain detailed information of P1-, P2-, and P3-specificities of this enzyme. And we also measured kinetic parameters of three commercially available enzymes, proteinase K, subtilisin BPN’, and subtilisin Carlsberg, the substrate specificities of which are already known,13,14,16,18 and compared the parameters of aqualysin I with those of three enzymes.

Materials and Methods

Enzymes. Aqualysin I was purified from the culture medium of *Thermus aquaticus* YT-1 according to the method described previously.23 Proteinase K (Merck, lots 710 E609668), subtilisin Carlsberg (Sigma Chemical Co., No. P-5380, lots 18F-0005) and subtilisin BPN’ (Nagase Biochemicals. Ltd., Bacterial A1-Protease Nagarse, lots 6928013) were purchased, and were further purified by column chromatography using an FPLC system equipped with a Mono-S column.

Chromogenic peptides. The chromogenic tripeptides, succinyl-Ala-Ala-Ala-p-nitroanilide, succinyl-Ala-Pro-Ala-p-nitroanilide, and succinyl-Ala-Ala-Val-p-nitroanilide were purchased from Sigma Chemical Co. The p-nitroanilides of succinyl-Phe-Val-Ala, succinyl-Phe-Val-Leu, succinyl-Phe-Val-Phe, succinyl-Phe-Ala-Ala, succinyl-Phe-Leu-Ala, succinyl-Phe-Nle-Ala, and succinyl-Phe-Val-Ala were synthesized from p-nitroanilide derivatives of amino acids, step by step in liquid phase using the mixed anhydride method, adding tert-butoxycarbonyl acylated amino acids to elongate toward the amino-terminus through reactions. All derivatives of amino acids for synthesis were purchased from Kukusan Chemical Works Ltd. Succinyl-Ile-Val-Ala-p-nitroanilide, succinyl-Phe-β-Ala-Ala-p-nitroanilide, succinyl-Gly-β-Ala-Ala-p-nitroanilide, succinyl-Leu-β-Ala-Ala-p-nitroanilide, and succinyl-Ile-β-Ala-Ala-p-nitroanilide were kindly supplied by Dr. K. Asano, Kirin Brewery Co., Ltd, Pharmaceutical Laboratory. The chromogenic tetrapeptide succinyl-Ala-Ala-Pro-Phe-p-nitroanilide was purchased from Sigma Chemical Co.

Measurement of kinetic parameters. Each substrate was dissolved in HEPES buffer (100 mM HEPES, 1 mM

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Abbreviations: HEPES, N-(2-Hydroxyethyl)piperazine-N’-2-ethanesulfonic acid; FPLC, Fast Protein Liquid Chromatography; ε₄₁₀, Molar extinction coefficient at 410 nm; kₘₐ, Catalytic rate constant; Kₘₐ, Michaelis constant; Nle, Norleucine; Suc-, Succinyl-(3-Carboxypropyl)-; pNA, p-nitroanilide or p-nitroanilide
CaCl₂, pH 7.5 at 40°C) over the solubility limit of the reagent prior to 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 (ε₄₈₀ = 8680 cm⁻¹ M⁻¹) after complete hydrolysis by a high concentration of enzyme or by alkali. Each reaction was started by addition of enzyme solution (30 μl) to substrate solution (270 μl) in a quartz cell on a spectrophotometer with a thermostated cell compartment, then the release of p-nitroaniline was monitored at 410 nm. Spontaneous hydrolysis of all chromogenic peptides was small enough within experimental error. Kinetic parameters, kcat and Km, were calculated from initial rate measurements for hydrolysis of p-nitroanilide substrates, by fitting to the Michaelis-Menten equation using a nonlinear regression algorithm.

Results

Subsies of aqualysis I

To examine the substrate specificity of aqualysis I, we synthesized some chromogenic tripeptides and calculated the kinetic parameters. The enzyme concentrations used for the hydrolyses of suc-Phe-Ala-Ala-pNA, suc-Phe-Nle-Ala-pNA, suc-Phe-Leu-Ala-pNA, suc-Phe-Val-Ala-pNA, suc-Ile-Val-Ala-pNA, suc-Ala-Ala-Ala-pNA and suc-Ala-Ala-Phe-pNA were around 10–100 nm. The enzyme concentrations used for the hydrolyses of suc-Phe-Val-Leu-pNA, suc-Ala-Ala-Val-pNA, suc-Ala-Pro-Ala-pNA and suc-Gly-Gly-Phe-pNA were around 0.5–2.0 μM.

Kinetic parameters for the hydrolysis of succinyltripeptide-p-nitroanilide are summarized in Table 1. Most polyptides were cleaved by aqualysis I. Efficient hydrolyses were observed with the substrate suc-Phe-X-Ala-pNA (X = alanine, norleucine, valine, and leucine) and suc-Ile-Val-Ala-pNA. The most efficient cleavage was observed with suc-Phe-Ala-Ala-pNA. The hydrolyses of suc-Phe-Val-Phe-pNA, suc-Phe-βAla-Ala-pNA, suc-Leu-βAla-Ala-pNA, suc-Ile-βAla-Ala-pNA, and suc-Gly-βAla-Ala-pNA were not detected, even in the presence of 10 μM concentration of enzyme. The proteolytic efficiency depended on the amino acid sequence of the substrate. Tripeptide, corresponding to P1, P2, and P3 sites, was important for the substrate-recognition of the enzyme. These results indicated that there existed at least three subsites, S1, S2, and S3, within the substrate binding site of aqualysis I.

### P1-specificity of aqualysis I

Sixteen tripeptides were tested, and the kinetic parameters of eleven peptides were measured (Table 1). The value of kcat for suc-Phe-Val-Ala-pNA was about 70 times that for suc-Phe-Val-Leu-pNA. The Michaelis constant for the latter substrate was about 10 times that for former one. These results showed that the S1 site of aqualysis I preferred alanine, leucine, and phenylalanine in this order. In this case, the amino acid positioned in the P1 site effected both kcat and Km values. The value of kcat for suc-Ala-Ala-Ala-pNA was about 30 times that for suc-Ala-Ala-Val-pNA. The Michaelis constant for the former substrate was identical to the latter one. These results showed that S1 site of aqualysis I preferred alanine to valine. In this case, the amino acid positioned in the P1 site affected only the kcat value. The results from studies on suc-Phe-Val-X-pNA, where X represents for alanine and phenylalanine, differed from those on suc-Ala-Ala-X-pNA. The value of kcat for suc-Ala-Ala-Phe-pNA was about 2 times that for suc-Ala-Ala-Ala-pNA. Aqualysis I hydrolyzed the former substrate more efficiently than the latter one, with a smaller value of Km. These results showed that the S1 site of aqualysis I preferred alanine and phenylalanine residues to valine and leucine residues. The P1-specificity of this enzyme was dependent on the kinds of P2 and P3 amino acid residues.

### P2-specificity of aqualysis I

Aqualysis I hydrolyzed efficiently suc-Phe-X-Ala-pNA, where X represents for alanine, norleucine, valine, and leucine. The Michaelis constants for suc-Phe-Ala-Ala-pNA, suc-Phe-Nle-Ala-pNA, and suc-Phe-Val-Ala-pNA were almost identical. The Michaelis constant for suc-Phe-Leu-Ala-pNA was about two times those above described. The value of kcat for suc-Phe-Ala-Ala-pNA was slightly larger than that for suc-Phe-Nle-Ala-pNA, and was about two times those for suc-Phe-Val-Ala-pNA and suc-Phe-Leu-Ala-pNA. In this case, the S2 site of aqualysis I preferred alanine, norleucine, valine, and leucine in this order. Norleucine is an analog of methionine, replacing the sulfur atom with a methylene group. Valine and leucine are more bulky than alanine and norleucine around the β- or γ-carbon of the side chain. These results suggested that the cavity of S2 site of aqualysis I was wide enough for alanine and norleucine but was narrow for efficient accepting of valine and leucine.

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**Table 1.** Substrate Specificity of Aqualysis I.

<table>
<thead>
<tr>
<th>P3</th>
<th>P2</th>
<th>P1</th>
<th>kcat [sec⁻¹]</th>
<th>Km [M]</th>
<th>kcat/Km [sec⁻¹ M⁻¹]</th>
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<td>Ala</td>
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<td>4.0 × 10⁻⁴</td>
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<tr>
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<td>Ala</td>
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<td>ND</td>
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<td>Val</td>
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<tr>
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<td>Ala</td>
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<td>1.6 × 10⁻⁵</td>
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<td>7.9 × 10⁻⁴</td>
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<tr>
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<td>Phe</td>
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<tr>
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<td>1.6 × 10⁻³</td>
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<td>Ala</td>
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</tr>
<tr>
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<td>βAla</td>
<td>Ala</td>
<td>ND</td>
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</tr>
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</table>

ND: Not detected.
Aqualysin I did not hydrolyze efficiently proline-containing peptide in the position of P2 site. Glycine-containing substrate suc-Gly-Gly-Phe-pNA was hydrolyzed with lower efficiency by aqualysin I than alanine-containing substrate suc-Ala-Ala-Phe-pNA. Cleavages of β-alanine-containing peptides were not detected.

These results showed that peptides comprised of only α-amino acid residues, positioned P1, P2, and P3, were recognized as substrates by aqualysin I.

**P3-specificity of aqualysin I**

Aqualysin I hydrolyzed suc-Phe-Val-Ala-pNA and suc-Ile-Val-Ala-pNA efficiently. The values of \(k_{cat}\) for these substrates were almost identical. The Michaelis constant for the former substrate was slightly smaller than that for latter one. The S3 site of aqualysin I preferred both phenylalanine and isoleucine residues.

The value of \(k_{cat}\) for suc-Phe-Ala-Ala-pNA was about seven times that for suc-Ala-Ala-Ala-pNA. The Michaelis constant for the latter substrate was about three times that for the former one. Aqualysin I hydrolyzed phenylalanine-containing substrates more efficiently than alanine-containing substrates in the position of the P3 site. The S3 site of aqualysin I preferred phenylalanine to alanine.

These results showed that the S3 site of aqualysin I preferred bulky hydrophobic residues.

**Substrate specificity of proteinase K, subtilisin Carlsberg, and subtilisin BPN’**

The substrate specificities of subtilisin BPN’, subtilisin Carlsberg, and proteinase K are already known, however these substrate specificities were measured under the arbitrary conditions, using peptides in different sequences. We analyzed substrate specificities of these enzymes under the standard assay conditions for aqualysin I, using the same peptides, to compare the specificities of these enzymes with that of aqualysin I.

Eight tripeptides were prepared to examine the substrate specificities of proteinase K and subtilisin Carlsberg. Seven tripeptides were prepared to examine the substrate specificity of subtilisin BPN’.

The enzyme concentrations of proteinase K used for the hydrolyses of suc-Phe-X-Ala-pNA (X=alanine, norleucine, valine, and leucine), suc-Ile-Val-Ala-pNA and suc-Ala-Ala-Ala-pNA were about 20 nm, and those for suc-Phe-Val-Phe-pNA and suc-Ala-Ala-pNA were around 1–7 μM. The enzyme concentrations of subtilisin Carlsberg used for the hydrolyses of suc-Phe-X-Ala-pNA (X=norleucine, valine and leucine) and suc-Ile-Val-Ala-pNA were about 20 nm, and those for suc-Phe-Ala-Ala-pNA, suc-Ala-Ala-Ala-pNA, and suc-Phe-Val-Leu-pNA were around 50–100 nm. The enzyme concentration for the hydrolysis of suc-Ala-Ala-Val-pNA was about 1 μM. The enzyme concentrations of subtilisin BPN’ used for the hydrolyses of suc-Phe-X-Ala-pNA (X=alanine, norleucine, valine and leucine), and suc-Ile-Val-Ala-pNA, suc-Ala-Ala-Ala-pNA, and suc-Phe-Val-Leu-pNA were about 50 nm, and that for the hydrolysis of suc-Ala-Ala-Val-pNA was about 5 μM. All peptides tested were cleaved by these enzymes and the kinetic parameters were calculated (Table 2).

Proteinase K cleaved suc-Phe-X-Ala-pNA (X=alanine, norleucine, valine and leucine), suc-Ile-Val-Ala-pNA, and suc-Ala-Ala-Ala-pNA efficiently. The value of \(k_{cat}\) for suc-Phe-Ala-Ala-pNA was slightly larger than that for suc-Phe-Nle-Ala-pNA, and was about five times those for suc-Phe-Val-Ala-pNA and suc-Phe-Leu-Ala-pNA. The Michaelis constant for suc-Phe-Ala-Ala-pNA was almost identical with that for suc-Phe-Nle-Ala-pNA. The value of \(k_{cat}\) for each of these substrates as well as the \(K_m\) of this enzyme were almost identical with that of aqualysin I. The S2 site of proteinase K preferred alanine, norleucine to valine and leucine, as shown in the case of aqualysin I. The value of \(k_{cat}\) for suc-Phe-Val-Ala-pNA was about three times that for suc-Phe-Val-Leu-pNA. The value of \(k_{cat}\) for suc-Ala-Ala-Ala-pNA was 450 times that for suc-Ala-Ala-Val-pNA. The Michaelis constant for the former substrate was identical with that for latter one. The S1 site of proteinase K preferred alanine to leucine and valine. On the whole, the substrate specificity of proteinase K was simi-
lar to that of aqualysin I.

Subtilisin Carlsberg cleaved suc-Phe-X-Ala-pNA (X = alanine, norleucine, valine, and leucine), suc-Ile-Val-Ala-pNA, suc-Phe-Val-Leu-pNA, and suc-Ala-Ala-Ala-pNA efficiently. The S3 site preferred both phenylalanine and isoleucine. Most efficient cleavage was observed with suc-Ile-Val-Ala-pNA. The value of $k_{\text{cat}}$ for this substrate by subtilisin Carlsberg was eight times that by aqualysin I and five times that by proteinase K. Subtilisin Carlsberg hydrolyzed this substrate more efficiently than proteinase K and aqualysin I. Comparison of parameters for the hydrolysis of suc-Phe-X-Ala-pNA (X = alanine, norleucine, valine, and leucine) showed that the S2 site of subtilisin Carlsberg preferred norleucine, leucine, and valine to alanine, while the S2 sites of aqualysin I and also proteinase K preferred alanine to other hydrophobic residues. The P1-specificity of subtilisin Carlsberg was also different from those of aqualysin I. Subtilisin Carlsberg cleaved suc-Phe-Val-Leu-pNA efficiently. The value of $k_{\text{cat}}$ for this substrate by subtilisin Carlsberg was about 90 times that by aqualysin I, and was about 10 times that by proteinase K. The value of $k_{\text{cat}}$ for suc-Ala-Ala-Val-pNA was about three times that by aqualysin I, and about 27 times that by proteinase K. The S1 site of subtilisin Carlsberg preferred alanine, but was also tolerant to bulky residues, valine and leucine. On the whole, substrate specificity of subtilisin Carlsberg was different from that of aqualysin I.

Subtilisin BPN' cleaved suc-Phe-X-Ala-pNA (X = alanine, norleucine, valine, and leucine) and suc-Ala-Ala-Ala-pNA efficiently. The S3 site preferred phenylalanine, as shown in the case of aqualysin I and other enzymes. The most efficient cleavage was observed with suc-Phe-Ala-Ala-pNA. The value of $k_{\text{cat}}$ for this substrate was about three times those for suc-Phe-Nle-Ala-pNA, suc-Phe-Val-Ala-pNA, and suc-Phe-Leu-Ala-pNA. The S2 site of subtilisin BPN' preferred alanine to other hydrophobic residues. The value of $k_{\text{cat}}$ for this substrate by subtilisin BPN' was about four times that by subtilisin Carlsberg. Subtilisin BPN' hydrolyzed this substrate more efficiently than subtilisin Carlsberg. Comparison of parameters for suc-Ala-Ala-Ala-pNA and suc-Ala-Val-Ala-pNA, and also suc-Phe-Val-Ala-pNA and suc-Phe-Val-Leu-pNA showed that the S1 site of subtilisin BPN' preferred alanine to leucine and valine, like aqualysin I and proteinase K. The substrate specificity of subtilisin BPN' was similar to those of aqualysin I and proteinase K, and was different from that of subtilisin Carlsberg.

The substrate specificities of subtilisin BPN', subtilisin Carlsberg and proteinase K were consistent with those previously reported. Subtilisin Carlsberg and proteinase K were also expected to exist, because crystallographic and kinetic studies on proteinase K and subtilisins suggested that these proteases contain S4 and S5 sites in the substrate binding site. We examined the proteolytic activity of aqualysin I using a chromogenic tetrapeptide succ-Ala-Ala-Pro-Phe-pNA to confirm the existence of S4 and S5 sites of aqualysin I. This commercially available peptide is often used as a standard substrate for subtilisin BPN' [8,9,11,13,14]. Kinetic parameters are summarized in Table 3. Every protease hydrolyzed this substrate with high efficiency. Although this substrate contains proline in the position of P2 site and alanine in the position of P3 site, the value of $k_{\text{cat}}$ for this substrate by aqualysin I was about three times that for succ-Phe-Ala-Ala-pNA, the most efficiently cleaved tripeptide substrate. Same tendencies were observed with other enzymes. This tetrapeptide substrate was more efficiently hydrolyzed than other tripeptide substrate tested. These results suggested that aqualysin I had S4 and S5 sites. Electrostatic interactions between succinyl group in the position of P5 site and S5 site may contribute to the efficient hydrolysis of this tetrapeptide.

**Discussion**

Many structural and kinetic studies on subtilisins and proteinase K have shown that the substrate binding sites of subtilisin-related enzymes are comprised of several subsites, including S1, S2, and S3 sites [8-16]. The preliminary kinetic analysis on aqualysin I suggested that the subsites, S1, S2, and S3, might exist within the substrate binding site of aqualysin I. Kinetic analyses on aqualysin I using tripeptide substrates found that aqualysin I was comprised of three subsites, S1, S2, and S3 sites. The S1 site preferred alanine and phenylalanine to valine and leucine. There was no proportional relationship between the value of $k_{\text{cat}}$ and the hydrophobicity of the P1 amino acid. Furthermore, there was no proportional relationship between the value of $k_{\text{cat}}$ and the volume of the P1 amino acid. Recognition system of P1 residue of aqualysin I will be a further problem.

The S2 site of aqualysin I preferred alanine and norleucine to valine and leucine. Crystallographic analyses on proteinase K and subtilisins showed that the S2 site of these enzymes forms a half-sphere-shaped pocket, making a narrow cleft. This cleft seems to be wide enough for accepting alanine and norleucine, but is narrow for valine and leucine. We suppose that the P2 specificity of aqualysin I is defined by the volume and

<table>
<thead>
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<th>enzyme</th>
<th>$k_{\text{cat}}$ [sec$^{-1}$]</th>
<th>$K_{\text{m}}$ [M]</th>
<th>$k_{\text{cat}}/K_{\text{m}}$ [sec$^{-1}$ M$^{-1}$]</th>
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<td>Aqualysin I</td>
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<td>Subtilisin BPN'</td>
<td>$4.8 \times 10^2$</td>
<td>$2.9 \times 10^{-4}$</td>
<td>$1.7 \times 10^6$</td>
</tr>
</tbody>
</table>

**Hydrolysis of tetrapeptide substrate**

Those results showed that aqualysin I had subsites, S1, S2, and S3. The S4 and S5 subsites of aqualysin I were also expected to exist, because crystallographic and kinetic studies on proteinase K and subtilisins suggested that these proteases contain S4 and S5 site in the substrate binding site. We examined the proteolytic activity of aqualysin I using a chromogenic tetrapeptide succ-Ala-Ala-Pro-Phe-pNA to confirm the existence of S4 and S5 sites of aqualysin I. This commercially available peptide is often used as a standard substrate for subtilisin BPN'. Kinetic parameters are summarized in Table 3. Every protease hydrolyzed this substrate with high efficiency. Although this substrate contains proline in the position of P2 site and alanine in the position of P3 site, the value of $k_{\text{cat}}$ for this substrate by aqualysin I was about three times that for succ-Phe-Ala-Ala-pNA, the most efficiently cleaved tripeptide substrate. Same tendencies were observed with other enzymes. This tetrapeptide substrate was more efficiently hydrolyzed than other tripeptide substrate tested. These results suggested that aqualysin I had S4 and S5 sites. Electrostatic interactions between succinyl group in the position of P5 site and S5 site may contribute to the efficient hydrolysis of this tetrapeptide.

The S2 site of aqualysin I preferred alanine and norleucine to valine and leucine. Crystallographic analyses on proteinase K and subtilisins showed that the S2 site of these enzymes forms a half-sphere-shaped pocket, making a narrow cleft. This cleft seems to be wide enough for accepting alanine and norleucine, but is narrow for valine and leucine. We suppose that the P2 specificity of aqualysin I is defined by the volume and
the width of S2 site pocket.

The S3 site of each enzyme tested preferred bulky, hydrophobic amino acid residues, phenylalanine and isoleucine. Crystallographic analyses on subtilisins and proteinase K suggested that there is no pocket or cleft to accept the side chain of the amino acid residue in the position of P3 site, and the side chain of the P3 amino acid is exposed to the solvent molecules, directed toward the outside of the enzyme.\textsuperscript{9,11,12} We suppose that the enzyme surface of aqualysin I itself acts as the S3 site pocket, which can accept even a bulky side chain of P3 amino acid.

From results for tetrapeptide substrate, we suppose that aqualysin I includes the S4 site as well as S1, S2, and S3 sites, and the binding between the S4 site and the substrate may contribute to the hydrolytic efficiency. Analysis on recognition system of P4-specificity of aqualysin I will be also a further problem.

On the whole, the substrate specificity of aqualysin I was similar to those of subtilisin BPN’ and proteinase K. The structures of these enzymes may be available as a suitable structure model of aqualysin I for designing of substrate specificity of this enzyme.

Acknowledgment
We are greatly thankful for Mr. Motoyoshi Nomizu and Dr. Katsuhiko Asano at KIRIN Brewery Co., Ltd., Pharmaceutical Laboratory, who have kindly instructed us the technique of peptide substrate synthesis.

References