Engineering of S2 Site of Aqualysin I; Alteration of P2 Specificity by Excluding P2 Side Chain

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ABSTRACT: Gly^{101}, one of the conserved amino acid residues which was expected to be comprised in half-sphere-shaped S2 site small pocket of aqualysin I, a microbial thermophilic alkaline serine protease, was replaced by alanine, valine, or leucine to alterate the P2 specificity of the enzyme by excluding bulky P2 side chain of the substrate. By the mutation of G101A, the catalytic efficiencies of the enzyme for bulky amino acid residues in P2 site such as valine and leucine drastically decreased by excluding the P2 side chain. By the mutation of G101V, even the side chain of the methyl group of the alanine and the side chain of proline were excluded, while the catalytic efficiency toward glycine residue was retained. The enzyme was altered to be glycine preferable. The mutation of G101L reduced catalytic efficiencies for any substrate including glycine which is corresponding to the main chain of the peptide substrate. The strategies we have adopted in this paper are applicable to all subtilisin-related enzymes.

Recently, many attempts on protein engineering have been made by using site-directed mutagenesis technique, for example, introduction of thermostability to a protein (*1*−*5*) and alteration of substrate specificity of an enzyme (*6*−*13*). Many attempts have been done using a microbial protease, subtilisin BPN′, one of the famous enzymes which have been often used as a model enzyme (*14*). Crystallographic as well as kinetic analyses on subtilisin BPN′ and its related enzymes show that there are some subsites, at least S1, S2, S3, and S4 sites, within the substrate-binding site of the enzyme (*15*−*29*). Crystallographic studies also show that the S1 and S2 sites form half-sphere-shaped pockets on the surface of the protein. However, the attempts on engineering of substrate specificity have been concerned mainly with the S1 site (*6*−*13*).

Engineering of S2 site, engineering of P2 specificity, is also important to design the specificity of a protease which hydrolyzes the specific sequence of peptide. Compared with the S1 site of subtilisin, the S2 site pocket is small and narrow to accept bulky amino acid residues such as phenylalanine. This time, we have decided to create a new glycine-specific protease by alteration of P2 specificity. As an engineered enzyme is often expected to be available for commercial use, which needs high level of stabilities, we have decided to do this engineering on a thermostable protease aqualysin I, one of subtilisin-related enzymes.

Aqualysin I is the alkaline serine protease isolated from prokaryotic origin *Thermus aquaticus* YT-1, an extreme thermophile (*30*−*39*). The gene encoding for this enzyme was cloned, and its amino acid sequence was determined. The primary structure of mature protein is homologous to those of *Bacillus* subtilisins and fungus proteinase K (34) (the homologous identities are around 40%). Aqualysin I has four cystein residues, like proteinase K, making two disulfide linkages (*33*), which are expected to contribute to the thermostability of aqualysin I. This protease displays broad specificity for cleavage of insulin B-chain (*31*) and hydrolyzed synthetic chromogenic peptides such as suc-tripeptide-pNA[1] (*39*). Results from these studies indicated that there exist subsites S1, S2, and S3 within the substrate binding site of aqualysin I.

Alteration of subsites of an enzyme whose tertiary structure is well-known may be easy. The tertiary structures of substrate-binding site of subtilisin BPN′, subtilisin Carlsberg, and proteinase K are identical enough to be superimposed each other (*15*−*29*). However, we have little information about the tertiary structure of aqualysin I. Prior to molecular designing of aqualysin I, we have assumed that the structure of the substrate-binding site of aqualysin I is identical with those of subtilisins. Comparative studies on aqualysin I with subtilisin BPN′, subtilisin Carlsberg and proteinase K under the same conditions supported this assumption (*38*−*39*). Therefore, we have employed tertiary structures of subtilisin BPN′, subtilisin Carlsberg, and proteinase K as suitable structural models for aqualysin I. Also, the engineering of S3 site of aqualysin I based on the structural homologies also supported the assumption (unpublished experiments).

Molecular replacement of all amino acid residues within or around the S2 site was done using computer graphics, and we have obtained Gly^{101} as a candidate. The side chain of alanine, valine, or the leucine residue, localized in the

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[1] Abbreviations: suc-, succinyl; -pNA, -*p*-nitroanilide; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; k_{cat}, catalytic rate constant; K_m, Michaelis constant.

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Table 1: Oligonucleotides Used for Site-Directed Mutagenesis[a]

mutant	oligonucleotide
G101A	5′-GTCCTGGACTGCAACGCGTCCGGCTCCACCTCT-3′ (inserts *Mlu*I site)
G101V	5′-GTCCTGGACTGCAACGTATCCGGCTCCACCTCT-3′
G101L	5′-GTCCTGGACTGCAACCTGTCCGGCTCCACCTCT-3′

[a] Underline indicates base changes from the pAQNΔC (wild-type) template.

position of Gly[101] introduced by molecular replacement, was expected to interact with P2 side chain.

In this paper, molecular replacement of Gly[101] of aqualysin I by alanine, valine, or leucine was done, and the P2 specificity of these mutant enzymes will be reported.

MATERIALS AND METHODS

Molecular Simulations. We have mainly utilized the tertiary structures of subtilisin Carlsberg and its inhibitor eglin c (PDB ID code, 2SEC). Most of simulations were performed on these structures first, and simulations on subtilisin BPN′ (PDB ID code, 1SBT) and proteinase K (PDB ID code, 2PRK) were followed. Computer simulations were done on a "micro-Vax 2000" system equipped with a PS-390 displaying element and with a program "biograf". Correcting operation of the orientation of introduced side chain was done with "molecular mechanics" protocols packaged within the application.

Enzymes. Wild-type aqualysin I was purified from the culture medium of *T. aquaticus* YT-1 according to the method described previously (*31*). Mutant enzymes, G101A, G101V, and G101L, were prepared by site-directed mutagenesis. The oligonucleotides for site-directed mutagenesis were synthesized with an Applied Biosystems 381A DNA synthesizer, and monomers for its synthesis were purchased from Applied Biosystems, Inc. (Table 1). Site-directed mutagenesis was done with M13mp19, using a Muta-Gene in vitro mutagenesis kit (Bio-Rad), as described by Kunkel et al. (*45*). The entire region of the DNA fragment was sequenced to prove that only the mutation expected had occurred. The protein expression vector, pAQNΔC, carrying mutant aqualysin I, was constructed with restriction sites. The mutant aqualysin I genes were expressed under the control of the *tac* promoter in *E. coli* MV1184 cells as described previously (*34*). Mutant enzymes were purified from *E. coli* cells according to the method described previously (*35*).

Chromogenic Peptides. The chromogenic peptides, suc-Ala-Ala-Ala-pNA and suc-Ala-Ala-Pro-Phe-pNA, were purchased from Sigma Chemical Co. The *p*-nitroanilide of suc-Phe-Leu-Ala, suc-Phe-Val-Ala, suc-Phe-Nle-Ala, and suc-Phe-Ala-Ala were synthesized from *p*-nitroanilide derivatives of amino acids, adding *tert*-butoxycarbonyl acylated amino acids to elongate toward amino-terminus through reactions, step by step in liquid phase using mixed anhydride method (*39*). All derivatives of amino acids for synthesis were purchased from Kokusan Chemical Works Ltd.

Determination of Kinetic Parameters. Each substrate was dissolved in HEPES buffer (100 mM HEPES and 1 mM $CaCl_2$, 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. Substrate concentration was determined spectrophotometrically from the absorbance of released *p*-nitroaniline (ϵ_{410} = 8680 cm^{-1} M^{-1}) after complete hydrolysis by the enzyme or by the alkali. Reactions were started by addition of enzyme solution (30 μL) to substrate solution (270 μL) in a quartz cell on a spectrophotometer equipped with a thermostated cell compartment, then the release of *p*-nitroaniline was monitored at 410 nm. Spontaneous hydrolysis of all peptides was small enough within experimental error. Kinetic parameters, k_{cat} and K_m, were determined from the initial rate measurements for hydrolysis of *p*-nitroanilide substrates, by fitting to the Michaelis−Menten equation using a nonlinear regression algorithm.

RESULTS

Molecular Simulations. Gly[101] of aqualysin I is conserved in subtilisins (corresponding to Gly[100] of subtilisin BPN′, subtilisin Carlsberg and proteinase K; see Table 2). Computer graphics analysis shows that the amino acid residue, Gly[101], locates on the surface of the protein, exposed to the solvent molecules. Introduction of alanine, valine, or leucine to this position by molecular replacement was expected to be a good strategy (Figure 1). Computer graphics analysis predicted that the introduced side chain stretched out into the S2 site pocket, occupying the cavity. When the target glycine was replaced by alanine, there still remained enough room in the S2 pocket for accepting glycine residue, the main chain of

Table 2: Partial Alignment of Amino Acid Sequences Around the Target Glycine[a]

enzyme	amino acid residues
aqualysin I [b]	D C N G^{101} S G S
proteinase K [c]	D D N G^{100} S G Q
thermitase [d]	D N S G^{108} S G T
subtilisin *Amylosaccharitics* [e]	D S T G^{100} S G Q
subtilisin BPN' [f]	G A D G^{100} S G Q
subtilisin Carlsberg [g]	N S S G^{100} S G Q
subtilisin DY [h]	N S S G^{100} S G T

[a] Partial alignment of amino acid sequences of microbial alkaline serine proteinases. The numbered glycine residue(s) represent(s) for the target glycine residue of aqualysin I, or the corresponding residues of other proteinases. [b] Ref *32*. [c] Ref 40. [d] Ref *41*. [e] Ref *42*. [f] Ref *43*. [g] Ref *14*. [h] Ref *44*.

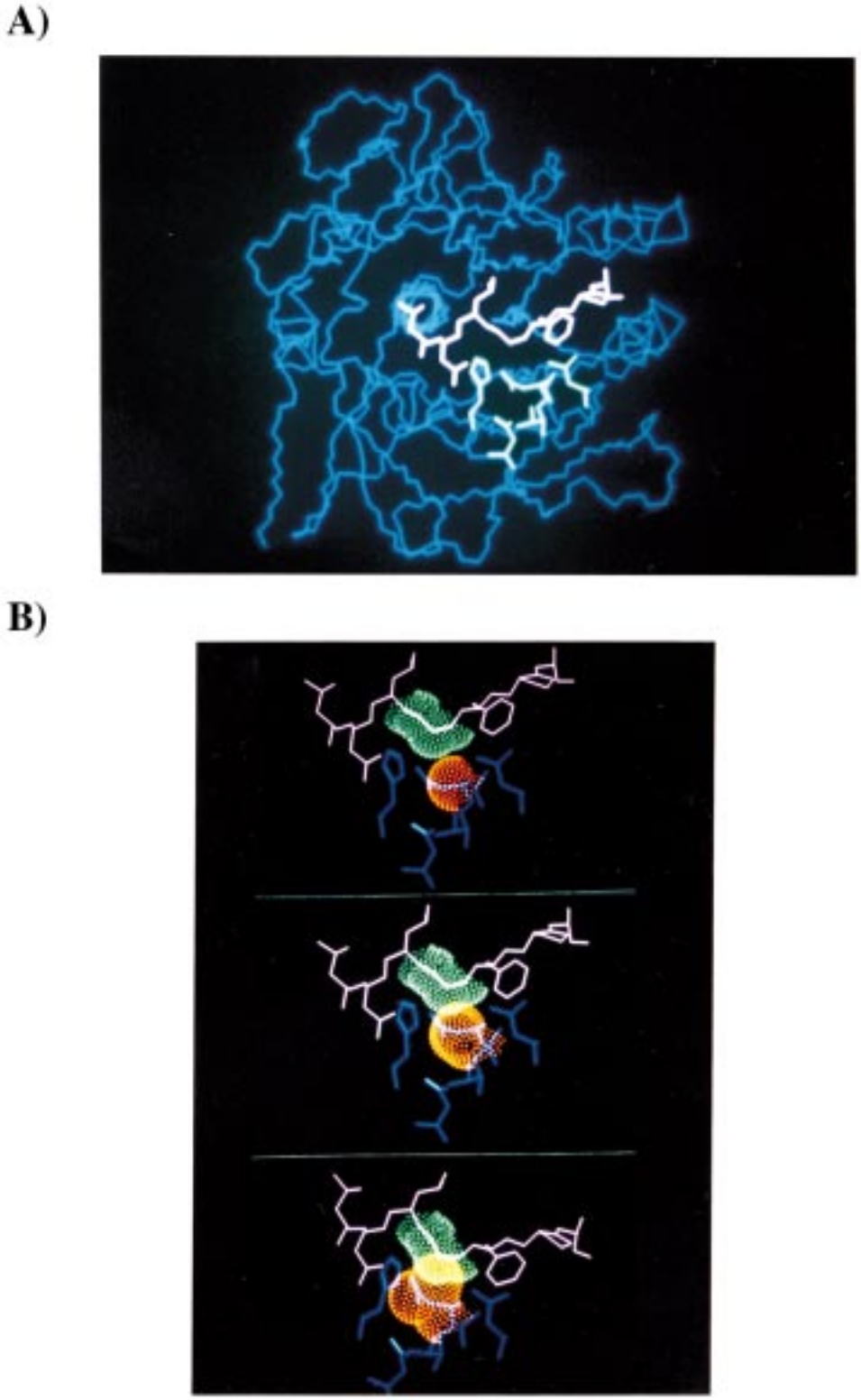

FIGURE 1: Graphic simulations of the main chain of subtilisin Carlsberg (in blue) and its S2 site residues (in green), and P2′−P5 fragment of the protease inhibitor, eglin c (in pink). The P2 residue of eglin c (proline) was replaced by glycine. (A) The whole structure of subtilisin Carlsberg. The main chain of the protein is in blue, and the side chains of S2 residues are in green. The S2 residues form a small half-sphere-shaped pocket. (B) Molecular simulations of amino acid replacements of the target glycine by alanine (upper), valine (middle), or leucine (lower). The S2 residues (in blue) and the P2′−P5 fragment of eglin c (in pink) are showed in wire-frame structure. van der Waals contact radii of the P2 glycine (in green) and the side chain of the replaced target residue (in red) are also showed. The introduced side chain stretches out into the S2 pocket, occupying the cavity. The S2 pocket with the alanine residue has still enough space to accept the glycine or main chain of the substrate (upper). While, the S2 pocket with the valine or leucine residue has little or no more space for the substrate.

the substrate. There was no room in the S2 pocket for accepting the main chain of the substrate when the target glycine was replaced by valine or leucine. These analyses predicted that we can alterate the P2 specificity of aqualysin

Table 3: Kinetic Analysis of S2 Site Mutants versus Substrates Having Variable P2 Residues[a]

	P2 residue											
	Leu			Val			Ala			Nle		
mutant	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
WT[b]	3.3	71	4.6×10^4	4.7	39	1.2×10^5	11	44	2.5×10^5	8.8	36	2.5×10^5
G101A[c]	*d*	>500[d]	2.7×10^1	0.18	1200	1.5×10^2	*d*	>500[d]	6.5	0.18	730	2.5×10^2
G101V[c]		ND[e]			ND[e]			ND[e]			ND[e]	
G101L[c]		ND[e]			ND[e]			ND[e]			ND[e]	

[a] Kinetic parameters for the hydrolyses of the tripeptide series suc-Phe-X-Ala↓pNA, where X was Leu, Val, Ala, or Nle. Assays were done at 40 °C, pH 7.5 (100 mM HEPES, 1 mM $CaCl_2$). Units are as follows: k_{cat}, s^{-1}; K_m, μM; and k_{cat}/K_m, M^{-1} s^{-1}. Standard errors were less than 18%. [b] Data from Tanaka et al. (39), in which assays were done under the same conditions of this work. [c] This work. [d] Parameters were not determined, because of large value of K_m over the solubility limit of the substrate. Only apparant second-order rate constant, k_{cat}/K_m, was determined. [e] Not detected.

Table 4: Kinetic Analysis of S2 Site Mutants versus Various Substrates[a]

	suc-Ala-Ala-Pro-Phe↓pNA			suc-Ala-Ala-Phe↓pNA			suc-Gly-Gly-Phe↓pNA		
mutant	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
WT[b]	33	1.2	2.7×10^4	2.5	0.91	2.8×10^3	0.15	1.6	9.5×10^1
G101A[c]	0.88	1.3	6.8×10^2	0.22	1.7	1.3×10^2	0.17	4.2	4.0×10^1
G101V[c]	0.90	0.73	1.2×10^3	0.091	1.0	9.0×10^1	0.25	3.4	7.2×10^1
G101L[c]	0.014	0.67	2.1×10^1	0.009	1.5	6.2	0.024	5.3	4.5

[a] Kinetic parameters for the hydrolyses of suc-tetrapeptide or -tripeptide↓pNA. Assays were done at 40 °C, pH 7.5 (100 mM HEPES, 1 mM $CaCl_2$). Units are as follows: k_{cat}, s^{-1}; K_m, mM; and k_{cat}/K_m, M^{-1} s^{-1}. Standard errors were less than 22%. [b] Data from Tanaka et al. (*39*), in which assays were done under the same conditions of this work. [c] This work.

I to glycine specific or glycine preferable by the replacement of Gly^{101} by alanine.

Kinetic Analysis of Variant Aqualysins. Four series chromogenic tripeptides, suc-Phe-X-Ala-pNA, where X was Leu, Val, Nle, or Ala, were tested with wild-type and three mutant enzymes. Assays were done at 40 °C, pH 7.5 (100 mM HEPES, 1 mM $CaCl_2$). The kinetic parameters are summarized in Table 3. Wild-type aqualysin I hydrolyzed these substrates efficiently; however, the catalytic efficiencies of mutant protein G101A drastically decreased. The kinetic parameters, k_{cat} and K_m, of suc-Phe-Leu-Ala-pNA and suc-Phe-Ala-Ala-pNA were not determined, because of the large value of K_m over the solubility limit of the peptides, and therefore, only apparent second-order rate constants k_{cat}/K_m were determined. The value of k_{cat} for suc-Phe-Val-Ala-pNA by G101A was about 4% of that by wild-type enzyme and the value of k_{cat} for suc-Phe-Nle-Ala-pNA by G101A was about 2% of that by wild-type enzyme. In every case, the value of K_m increased. These data suggested that the introduced side chain of alanine in the position of Gly^{101} excluded bulky side chain in the P2 site, by destabilizing the transition state as well as destabilizing the Michaelis complex formation. The hydrolyses of these peptides by G101V and G101L were not detected even in the presence of 10 μM enzyme. These results suggested that the introduced side chain to the position of Gly^{101} buried the S2 pocket, excluding the side chain of the P2 residue, and were consistent with the previous prediction by the computer simulations.

We tested, moreover, three peptides, suc-Ala-Ala-Phe-pNA, suc-Gly-Gly-Phe-pNA, and suc-Ala-Ala-Pro-Phe-pNA, to examine whether the mutant proteins prefer glycine or not. These commercially available peptides are not in series, but the P1 site is fixed to phenylalanine. We used these peptides, containing proline, alanine, or glycine in P2 site, as probes to analyze how the mutant protein recognized the P2 residue of the substrate. The kinetic parameters are summarized in Table 4.

(a) Proline as the P2 Residue. This peptide, suc-Ala-Ala-Pro-Phe-pNA, has been used as a standard substrate for assays of microbial serine proteases (*7*−*10*). Wild-type enzyme hydrolyzed this peptide substrate efficiently. However, the values of k_{cat} of mutant enzymes were reduced. The values of k_{cat} of both G101A and G101V were about 2.7% of that of wild-type enzyme. And the value of k_{cat} of G101L was drastically reduced. In every case, the magnitude of the values of K_m was around 1 mM, and no significant effects by the mutation to the binding of enzyme and the proline-containing substrate were observed. In every mutation, the introduced side chain seemed to have acted to exclude proline residue. These results indicated that the introduced side chain in the position of Gly^{101} destabilized the transition state, not the Michaelis complex formation, in the recognition of proline residue.

(b) Alanine as the P2 Residue. Wild-type enzyme showed efficient hydrolysis of this substrate suc-Ala-Ala-Phe-pNA. By introduction of bulky side chain in the position of Gly^{101}, the values of k_{cat} for the hydrolysis of this peptide were reduced. The value of k_{cat} of G101A was about 9% of that of wild-type enzyme, that of G101V was about 3.6%, and that of G101L was about 0.4%. As the volume of the side chain introduced to the position of Gly^{101} increased, the value of k_{cat} decreased. The values of K_m for this substrate remained around 1 mM, slightly affected by the mutations.

(c) Glycine as the P2 Residue. Wild-type as well as mutant proteins hydrolyzed the glycine-containing substrate suc-Gly-Gly-Phe-pNA, with lower efficiency than suc-Ala-Ala-Phe-pNA and suc-Ala-Ala-Pro-Phe-pNA. Wild-type aqualysin I hydrolyzed suc-Ala-Ala-Phe-pNA more efficiently than suc-Gly-Gly-Phe-pNA. The value of k_{cat} for former substrate was about 16 times that for latter substrate. The S2 site of wild-type enzyme preferred alanine more than the glycine residue. The parameters for suc-Gly-Gly-Phe-pNA by G101A and G101V were almost identical with those of wild-type enzyme. These results suggested that the introduced side chain of alanine or valine in the position of Gly^{101} interacted

only with the P2 side chain of a substrate to exclude β-carbon methyl group. The value of k_{cat} of G101L for suc-Gly-Gly-Phe-pNA was lowered, being about 16% of that by wild-type enzyme. In both cases, G101L displayed small amount of k_{cat} values.

Above results showed that mutant proteins G101V and G101L preferred the glycine-containing substrate to alanine-containing one. Although the proteolytic efficiencies for P2 glycine residue did not increase, these mutant proteins relatively preferred glycine. By the replacement of Gly^{101}, the P2 specificity of aqualysin I was altered.

DISCUSSION

Amino acid replacement of Gly^{101} of aqualysin I to alanine, valine, or leucine resulted in reduction of catalytic efficiency of the enzyme for the hydrolysis of peptide which contained the bulky amino acid residue in the position of P2 site. These results indicated that Gly^{101} lay within or on the wall of S2 pocket, and the introduced side chain in the position of Gly^{101} stretched out into the S2 pocket, occupying the cavity, as predicted by computer simulations. Replacement of Gly^{101} by alanine was sufficient to exclude a bulky amino acid residue in the position of P2 site, such as valine, leucine, and norleucine (Table 3). To exclude alanine and proline residues, replacement of Gly^{101} by valine was needed (Table 4). In this case, mutant protein G101V preferred glycine to alanine; however, the specificity was not so strict. The introduction of leucine to the position of Gly^{101} gave a drastic result. The S2 site of G101L excluded every amino acid residue, including glycine, corresponding to the main chain of the substrate.

Results also indicated that the recognition of the substrate was dependent on amino acid sequence of the substrate. The K_m values for suc-Ala-Ala-Pro-Phe-pNA, suc-Ala-Ala-Phe-pNA, and suc-Gly-Gly-Phe-pNA were not influenced by the kinds of amino acids introduced to the position of Gly^{101}, while the introduced side chain enlarged the k_{cat} values. On the other hand, the results of the hydrolyses of suc-Phe-X-Ala-pNA (X was Ala, Nle, Val, or Leu) were included both increasing K_m values and decreasing k_{cat} values. Recognition of alanine in P2 site was dependent on the amino acid sequence of the substrate (Table 4).

Compared to the S1 pocket, the volume of the S2 pocket is expected to be small. Crystallographic data predicted that the S1 pocket was large enough to accept a bulky amino acid residue such as phenylalanine, while the S2 pocket was too small and narrow to accept such amino acid residue (*17*, *18*). Removal of bulky side chain of some amino acid residues which comprise the wall of S2 site pocket is needed for alternation of the S2 site to accept bulky amino acid residues, which may disrupt the surroundings of active-site histidine (*9*). Therefore, the amino acid replacement around the active-site histidine should be additive in the volume of the side chain, as we have performed in this paper.

Gly^{101} of aqualysin I lies within the conserved region G^{101}-S^{102}-G^{103}, which is expected to form the part of the substrate binding cleft of the enzyme (Table 2). Because all molecular simulations were done using the structures of subtilisin and its related enzymes, the strategies we have adopted in this paper are applicable to all subtilisin-related enzymes. As all mutant enzymes in this paper, including heat-treatment steps in the purification procedures (*31*), were thermostable proteins, like wild-type aqualysin I, these proteins are also applicable to commercial use.

REFERENCES

1. Mitchinson, C., and Wells, J. A. (1989) *Biochemistry 28*, 4807−4815.
2. Wells, J. A., and Powers, D. B. (1986) *J. Biol. Chem. 261*, 6564−6570.
3. Katz, B. A., and Kassiakoff, A. (1986) *J. Biol. Chem. 261*, 15480−15485.
4. Pantoliano, M. W., Ladner, R. C., Bryan, P. N., Rollence, M. L., Wood, J. F., and Poulos, T. L. (1987) *Biochemistry 26*, 2077−2082.
5. Matsumura, M., and Matthews, B. W. (1989) *Science 243*, 792−794.
6. Khouri, H. E., Vernet, T., Menard, R., Parlati, F., Laflamme, P., Tessier, D. C., Gour-Salin, B., Thomas, D. Y., and Storer, A. C. (1991) *Biochemistry 30*, 8929−8936.
7. Estell, D. A., Graycar, T. P., Miller, J. V., Powers, D. B., Burnier, J. P., Ng, P. G., and Wells, J. A. (1986) *Science 233*, 659−663.
8. Wells, J. A., Powers, D. B., Bott, R. R., Graycar, T. P., and Estell, D. A. (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84, 1219−1223.
9. Carter, P., and Wells, J. A. (1987) *Science 237*, 394−399.
10. Russell, A. J., and Fersht, A. R. (1987) *Nature 328*, 496−500.
11. Takagi, H., Morinaga, Y., Ikemura, H., and Inouye, M. (1988) *J. Biol. Chem. 263*, 19592−19596.
12. Rheinnecker, M., Baker, G., Eder, J., and Fersht, A. R. (1993) *Biochemistry 32*, 1199−1203.
13. Ballinger, M. D., Tom, J., and Wells, J. A. (1995) *Biochemistry 34*, 13312−13319.
14. Smith, E. L., Delange, R. J., Evans, W. H., Landon, M., and Markland, F. S. (1968) *J. Biol. Chem. 243*, 2184−2191.
15. Betzel, C., Pal, G. P., and Saenger, W. (1988) *Eur. J. Biochem. 178*, 155−171.
16. Betzel, C., Pal, G. P., Struck, M., Jany, K.-D., and Saenger, W. (1986) *FEBS Lett. 197*, 105−110.
17. Wright, C. S. (1972) *J. Mol. Biol. 67*, 151−163.
18. Wright, C. S., Alden, R. A., and Kraut, J. (1972) *J. Mol. Biol. 66*, 283−289.
19. Robertus, J. D., Alden, R. A., Birktoft, J. J., Kraut, J., Powers, J. C., and Wilcox, P. E. (1972) *Biochemistry 11*, 2439−2449.
20. Poulos, T. L., Alden, R. A., Freer, S. T., Birktoft, J. J., and Kraut, J. (1976) *J. Biol. Chem. 251*, 1097−1103.
21. Bode, W., Papamokos, E., Musil, D., Seemueller, U., and Fritz, H. (1986) *EMBO J. 5*, 813−818.
22. Bode, W., Papamokos, E., and Musil, D. (1987) *J. Biol. Chem. 263*, 7895−7906.
23. Drenth, J., Hol, W. G. J., Jansonius, J. N., and Koekoek, R. (1972) *Eur. J. Biochem. 26*, 177−181.
24. Alden, R. A., Birktoft, J. J., Kraut, J., Robertus, J. D., and Wright, C. S. (1971) *Biochem. Biophys. Res. Commun. 45*, 337−344.
25. Heinz, D. W., Priestle, J. P., Rahuel, J., Wilson, K. S., and Grueter, M. G. (1991) *J. Mol. Biol. 217*, 353−371.
26. McPhalen, C. A., and James, M. N. (1988) *Biochemistry 27*, 6582−6598.
27. Kossiakoff, A. A., White, M. U., and Eigenbrot, C. (1991) *Biochemistry 30*, 1211−1221.
28. Gros, P., Kalk, K. H., and Hol, W. G. J. (1991) *J. Biol. Chem. 266*, 2953−2961.
29. Siezen, R. J, Vos, W. M., Leunissen, J. A. M., and Dijkstra, B. W. (1991) *Protein Eng. 4*, 719−737.
30. Matsuzawa, H., Hamaoki, M., and Ohta, T. (1983) *Agric. Biol. Chem. 47*, 25−28.
31. Matsuzawa, H., Tokugawa, K., Hamaoki, M., Mizoguchi, M., Taguchi, H., Terada, I., Kwon, S.-T., and Ohta, T. (1988) *Eur. J. Biochem. 171*, 441−447.
32. Kwon, S.-T., Terada, I., Matsuzawa, H., and Ohta, T. (1988) *Eur. J. Biochem. 173*, 491−497.

33. Kwon, S.-T., Matsuzawa, H., and Ohta, T. (1988) *J. Biochem. 104*, 557−559.
34. Terada, I., Kwon, S.-T., Miyata, Y., Matsuzawa, H., and Ohta, T. (1990) *J. Biol. Chem. 265*, 6576−6581.
35. Lee, Y.-C., Miyata, Y., Terada, I., Ohta, T., and Matsuzawa, H. (1991) *Agric. Biol. Chem. 55*, 3027−3032.
36. Lee, Y.-C., Ohta, T., and Matsuzawa, H. (1992) *FEMS Microbiol. Lett. 92*, 73−78.
37. Tanaka, T., Matsuzawa, H., and Ohta, T. (1998) *Biosci. Biotechnol. Biochem. 62*, 1806−1808.
38. Tanaka, T., Matsuzawa, H., Kojima, S., Kumagai, I., Miura, K., and Ohta, T. (1998) *Biosci. Biotechnol. Biochem. 62, 2035−2038*.
39. Tanaka, T., Matsuzawa, H., and Ohta, T. (1998) *Biosci. Biotechnol. Biochem. 62*, 2161−2165.
40. Gunkel, F. A., and Gassen, H. G. (1989) *Eur. J. Biochem. 179*, 185−194.
41. Meloun, B., Baudys, M., Frommel, C., and Hohne, W. E. (1985) *FEBS Lett. 183*, 195−200.
42. Stahl, M. L., and Ferrari, R. (1984) *J. Bacteriol. 158*, 411−418.
43. Vasantha, N., Thompson, L. D., Rhodes, C., Banner, C., Nagle, J., and Filpula, D. (1984) *J. Bacteriol. 159*, 811−819.
44. Nedkov, P., Oberthur, W., and Braunitzer, G. (1983) *Hoppe-Seyler's Z. Physiol. Chem. 364*, 1537−1540.
45. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods. Enzymol. 154*, 367.

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