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Identification and Designing of the S3 Site of Aqualysin I, a Thermophilic Subtilisin-Related Serine Protease

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Aqualysin I is a bacterial subtilisin-related alkaline serine protease, originating in *Thermus aquaticus* YT-1. Based on computational analysis, we predicted that two residues, Ser[102] and Gly[131], form the S3 site of aqualysin I, and we proved that this prediction by site-directed mutagenesis. To alter the P3-specificity of the enzyme, we built a "wall" on the S3 site edge by introducing a bulky side chain at target sites. Six mutant proteins were prepared: S102H, S102K, S102E, G131H, G131K, and G131D. The mutant enzymes were examined with two kinetically typical peptides for aqualysin I, suc-X-Ala-Ala-pNA, where X is Ala or Phe. All mutations reduced the efficiency for the Phe-containing peptide, while they raised the k_{cat} values for the Ala-containing peptide. Especially, the S102K mutant protein hydrolyzed the polyalanine peptide efficiently. The strategies we have adopted in this paper are applicable to all subtilisin-related enzymes.

Key words: aqualysin I, P3-specificity, S3 subsite, serine protease, subtilsin.

Recently, many attempts at protein engineering have been made using site-directed mutagenesis techniques, for example, promoting thermostability to a protein (*1-5*) or alterating the substrate specificity of an enzyme (*6-13*). Many attempts have been made using a microbial protease, subtilisin BPN′, one of a well-known group of enzymes which have often been used as model enzymes (*14*). These proteins contain subsites, among them the S1, S2, and S3 sites, that play important roles in substrate recognition. Crystallographic as well as kinetic analyses on subtilisin BPN′ and its related enzymes show that there are subsites, at least the S1, S2, S3, and S4 sites, within the substrate binding site of the enzyme (*15-29, 40-46*). Crystallographic studies also show that the S1, S2, and S4 sites form pockets on the surface or interior of the protein. Surprisingly, the S3 site has received little attention, only for the reason that the S3 site does not form a pocket. Attempts at engineering substrate specificity have concentrated on these pockets (*6-13*).

Engineering the P3-specificity of the S3 site, is also important in designing the specificity of a protease that hydrolyzes a specific peptide sequence. In contrast to the S1, S2, and S4 sites of subtilisin, the S3 site does not form a pocket. Structural studies have shown the side chain of the P3 residue to lie on the surface of the protein, exposed to the solvent (*15-29*). If the S3 site does not form a pocket, one possibility would be to build a wall near or within the S3 site to form a pocket.

For the present study, we decided to identify the S3 site in subtilisins and create new P3-specificity. Because an engineered enzyme is often expected to be available for commercial use, which requires that it be highly stable, we decided to use a thermostable protease, aqualysin I, a subtilisin-related enzyme.

Aqualysin I is an alkaline serine protease of prokaryotic origin isolated from *Thermus aquaticus* YT-1, an extreme thermophile (*30-41*). The gene encoding the enzyme was cloned and its amino acid sequence determined. The primary structure of the mature protein is homologous to those of *Bacillus* subtilisins and fungus proteinase K (*34*) (identities are around 40%). Aqualysin I has four cysteine residues, as in the case of proteinase K, that from two disulfide linkages (*33*) that are expected to contribute to the stability of this thermostable protein. The protease displays broad specificity for insulin B-chain (*31*), and hydrolyzes synthetic chromogenic substrates such as suc-tripeptide-pNA (*39, 40*). The results of these studies indicate that aqualysin I has subsites S1, S2, and S3 within the substrate binding site. The results also indicate that the S3 site prefers a bulky amino acid residue, for example, phenylalanine. For example, aqualysin I hydrolyzes suc-Phe-Ala-Ala-pNA more efficiently than the elastic substrate suc-Ala-Ala-Ala-pNA, with the k_{cat}/K_m value of the former substrate 200 times that of the latter substrate. If the P3-specificity of aqualysin I can be converted to "alanine specific," aqualysin I will become a poly-alanine specific protease like "elastase."

The alteration of a subsite on an enzyme whose tertiary structure is well-known may be easy. The tertiary structures of the substrate binding sites of subtilisin BPN′, subtilisin Carlsberg, and proteinase K are similar enough to

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

be superimposed on each other (*15-29*). However, little is known about the tertiary structure of aqualysin I. Prior to the molecular designing of aqualysin I, we assumed that the structure of the substrate binding site would be identical to those of subtilisins. Comparative kinetic studies of aqualysin I with subtilisin BPN′, subtilisin Carlsberg and proteinase K under the same conditions supported this assumption (*38, 39*). Moreover, the results of altering the P2-specificity of aqualysin I, done on the basis of this structural similarity, also supported this assumption (*41*). Therefore, we employed the tertiary structures of subtilisin BPN′, subtilisin Carlsberg and proteinase K as suitable structural models for aqualysin I.

The crystallographic studies of subtilisins show that the substrate binding cleft is formed mainly by glycine and serine residues (*15-29*); no bulky amino acid residues exist around the S3 site, while the side chain of the P3 amino acid residue is exposed to the solvent molecules and is directed toward the outside of the enzyme. To alter P3-specificity, an interactive and bulky side chain should be introduced onto the S3 site on the surface of the protein so as to form an S3 pocket.

Here, we chose Ser^{102} and Gly^{131} as the S3 site residues, and carried out the molecular replacement of Ser^{102} by histidine, lysine, or glutamic acid, and also the replacement of Gly^{131} by histidine, lysine, or aspartic acid. The substrate specificities of these mutant enzymes will be reported.

MATERIALS AND METHODS

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

Enzymes—Six single mutant enzymes, S102H, S102K, S102E, G131H, G131K, and G131D, were prepared by site-directed mutagenesis. The oligonucleotides for the site-directed mutagenesis were synthesized with an Applied Biosystems 381A DNA synthesizer using monomers purchased from Applied Biosystems (see Table I). Site-directed mutagenesis was done with M13mp19 using a Muta-Gene *in vitro* mutagenesis kit (Bio-Rad) as described by Kunkel *et al.* (*42*). The entire region of the DNA fragment was sequenced to prove that only the expected mutation had occurred. The protein expression vector, pAQNΔC, carrying mutant aqualysin I was constructed with the restriction sites. The mutant aqualysin I genes were expressed under the control of the *tac* promoter in *Escherichia coli* MV1184 cells as described previously (*34*). Mutant enzymes were purified from *E. coli* cells according to the method described previously (*35, 41*).

TABLE I. **Oligonucleotides used for site-directed mutagenesis.**

Mutant	Oligonucleotide
S102H	5′-CTGGACTGCAACGG<u>CCAT</u>GGCTCCACCTCTGGG-3′ (inserts *Nco*I site)
S102K	5′-CTGGACTGCAACGGT<u>AAA</u>GGCTCCACCTCTGGG-3′
S102E	5′-CTGGACTGCAACGGT<u>GAA</u>GGCTCCACCTCTGGG-3′
G131H	5′-AACATGAGCTTAGG<u>CCAT</u>GGAGTCTCCACTGCC-3′ (inserts *Nco*I site)
G131K	5′-AACATGAGCTTAGGT<u>AAA</u>GGAGTCTCCACTGCC-3′
G131D	5′-AACATGAGCTTAGGT<u>GAC</u>GGAGTCTCCACTGCC-3′

Base changes from the pAQNΔC template, the protein expression vector for the wild-type enzyme are underlined.

Chromogenic Peptides—The chromogenic peptides suc-Ala-Ala-Ala-pNA and suc-Ala-Ala-Pro-Phe-pNA were purchased from Sigma Chemical. Suc-Phe-Ala-Ala-pNA was synthesized in liquid phase using a mixed anhydride method as described previously (*38, 39*).

Determination of Kinetic Parameters—Before use, excess substrate was dissolved in HEPES buffer (100 mM HEPES, 1 mM $CaCl_2$, pH 7.5 at 40°C), 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} = 8,680 $cm^{-1} \cdot M^{-1}$) after complete hydrolysis by the enzyme or by alkali. Reactions were started by adding enzyme solution (30 μl) to the substrate solution (270 μl) in a quartz cell on a spectrophotometer equipped with a thermostatted cell compartment. The release of *p*-nitroaniline was monitored at 410 nm. The levels of spontaneous hydrolysis in all cases was small enough to be within experimental error. Kinetic parameters, k_{cat} and K_m, were determined from the initial rate measurements for the hydrolysis of *p*-nitroanilide substrates. Data were fitted to the Michaelis-Menten equation using a nonlinear regression algorithm as described previously (*39*).

RESULTS AND DISCUSSION

Molecular Simulations—In choosing candidate residues, we adopted two strategies: the side chain introduced should be directed toward the solvent, and the distance between the introduced side chain and the side chain of the P3 residue should be within 0.6 nm. The distance 0.6 nm is enough for the interaction of the P3 residue and the S3 site, and is also expected to exclude bulky P3 residues. Molecular replacement of all amino acid residues around the P3 residue was done using computer graphics, followed by simple energy optimization by correcting the orientation of the side chain. After molecular replacement simulations, we chose Ser^{102} (corresponding to Ser^{101} of subtilisin Carlsberg, Ser^{102} of proteinase K; see Table II) and Gly^{131} (corresponding to Gly^{128} of subtilisin Carlsberg, Gly^{135} of proteinase K) as the targets for molecular replacements.

Computer graphics analysis predicted that the two amino acid residues, Ser^{102} and Gly^{131}, are located on the surface of the protein, and that their introduced side chains are directed toward the outside of the enzyme (see Fig. 1). The distance between the β-carbon of Ser^{102} and the α-carbon of the P3 site residue is about 0.4 nm, and the distance between the α-carbon of Gly^{131} and the α-carbon of the P3 site residue is about 0.6 nm. Ser^{102} is closer to the P3 site.

The bulky side chain of the amino acid residue introduced in the position of Ser^{102} or Gly^{131} by molecular replacement may interact with the side chain of the P3 residue, and is expected to exclude the bulky phenylalanine side chain if this amino acid is positioned in the P3 site. We chose lysine,

which has a long side chain, histidine, with an imidazole ring side chain, and either acidic residue aspartic acid or glutamic acid, to introduce at the target positions, expecting that the hydrophilic side chain might be exposed to the solvent and interact with the P3 side chain.

Identification of the S3 Site and P3-Specificity—To examine whether the two target residues, Ser[102] and Gly[131], exist on or within the S3 of the enzyme, and also to examine whether the introduction of bulky side chains at these sites is sufficient to alter the P3-specificity of the enzyme, we prepared six mutant proteins: S102H, S102K, S102E, G131H, G131K, and G131D, and examined their P3-specificities with two kinds of peptides. Two peptides, suc-X-Ala-Ala-pNA, in which X is Ala or Phe, were used to

TABLE II. **Partial alignment of amino acid sequences of serine proteases around the target residues.**

Protease (origin)	Partial sequence around the target residues
aqualysin I (*T. aquaticus* YT-1)	--- CNG **Ŝ**[102] GST --- SKG **Ġ**[131] GVS ---
rt41A protease (*T.* rt41A)	--- CNG **S** GSN --- SLG **G** GAS ---
protease A (*V. alginolyticus*)	--- CSG **S** GST --- SLG **G** GQS ---
thermitase (*T. vulgaris*)	--- NSG **S** GTW --- SLG **G** TVG ---
subtilisin *Amylosacchariticus* (*B. Amylosacchariticus*)	--- STG **S** GQY --- SLG **G** PTG ---
subtilisin BPN′ (*B. amyloliquefaciens*)	--- ADG **S** GQY --- SLG **G** PSG ---
subtilisin Carlsberg (*B. licheniformis*)	--- SSG **S** GTY --- SLG **G** PSG ---
subtilisin DY (*B. subtilis* DY)	--- SSG **S** GTY --- SLG **G** PSG ---
subtilisin 168 (*B. subtilis* 168)	--- STG **S** GQY --- SLG **G** PTG ---
subtilisin BP92 (*B. alcalophilus* BP92)	--- ASG **S** GSV --- SLG **S** PSP ---
alkaline elastase YaB (*B. subtilis* YaB)	--- ASG **S** GSI --- SLG **S** SAG ---
subtilisin 147 (*B. lentis*)	--- RNG **S** GSL --- SLG **S** TSG ---
proteinase K (*T. album* Limber)	--- DNG **S** GQY --- SLG **G** GYS ---
alkaline protease (*A. chrysogenum*)	--- RGS **S** SST --- SLG **G** GYS ---
alkaline protease (*A. oryzae* ATCC20386)	--- QGE **S** SST --- SLG **G** GYS ---
protease B (*S. cerevisiae*)	--- SNG **S** GTM --- SLG **G** GKS ---
alkaline extracellular protease (*Y. lipolytica*)	--- AGR **S** AAL --- SGG **G** PKS ---

Amino acid residues indicated in bold-type represent the target Ser[102] and Gly[131] of aqualysin I, and the corresponding residues of other serine proteases, respectively. The partial alignment of amino acid sequences of subtilisin-type alkaline serine proteases were from a review (*29*).

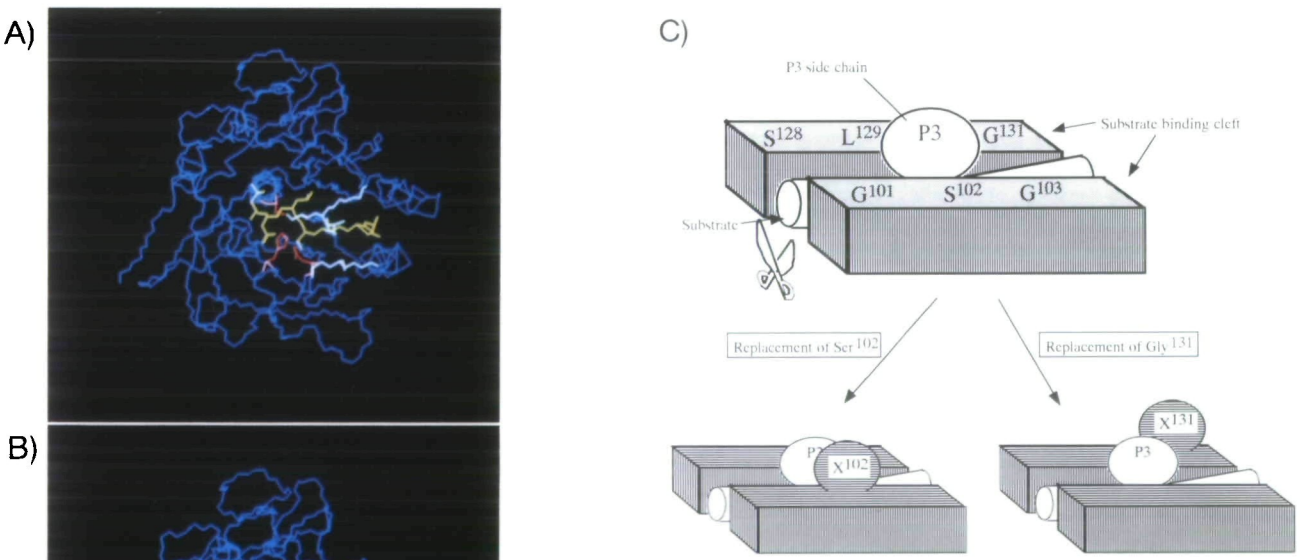

Fig. 1. **Schematic representation of the substrate binding cleft of the enzyme and the substrate.** (A) The whole structure of subtilisin Carlsberg (in blue) and the P2′-P5 fragment of the protease inhibitor, eglin c, is in yellow. The substrate binding strands, S^{125}-L^{126}-G^{127}-G^{128} and G^{100}-S^{101}-G^{102} (corresponding to S^{125}-L^{126}-G^{127}-G^{128} and G^{99}-S^{100a}-G^{101} of aqualysin I), of the enzyme are indicated in white, and the catalytic triad residues, Ser^{221}, His^{64}, and Asp^{32} (corresponding to Ser^{222}, His^{70}, and Asp^{39} of aqualysin I), are in red. B) The target Ser^{101} and Gly^{128} (in white) are indicated. The P2′-P5 fragment of the protease inhibitor, eglin c, is in green; the P3 residue was replaced by phenylalanine. C) Schematic representation of substrate binding cleft of the enzyme and the substrate. Upper figure shows the wild-type enzyme (hatched) with a bulky P3 amino acid residue (white). The main chain of the substrate (white) is located between two glycine-rich strands enzyme (hatched) forming anti-parallel β-sheet. "Scissors" lying left-side of the "cleft" represents for the active serine residue. The lower two figures represent the interaction between the side chain of the P3 residue and the bulky side chain on the enzyme introduced by molecular replacement at Ser^{101} or Gly^{128} of aqualysin I.

examine P3-specificity. Alanine and phenylalanine, small and large residues, respectively, were chosen as probes to examine the P3-specificity of the mutant enzymes because it was not feasible to test all kinds of peptides. The P1 and P2 sites of the peptide were fixed as alanine, because the wild-type enzyme prefers alanine as both the P1 and P2 residues (*39*). The kinetic parameters are summarized in Table III.

Wild-type aqualysin I prefers phenylalanine to alanine as the P3 residue (*39*), as shown in Table III, and hydrolyzes the phenylalanine-containing substrate much more efficiently. The k_{cat} for the phenylalanine-containing substrate is about 10 times that for the alanine-containing one, while the K_m for the former substrate is much smaller than that for the latter. The P3-specificities of the mutant proteins differed from that of the wild-type enzyme. The k_{cat} values for suc-Phe-Ala-Ala-pNA were reduced to 1-26%, while the k_{cat} values for suc-Ala-Ala-Ala-pNA were higher. These results suggest that the amino acid residues Ser^{102} and Gly^{131} lie on or within the S3 site, and that the side chains introduced at these positions interact with the side chain of the P3 residue. The results for S102K, G131H, and G131K show that the side chain introduced at the S3 site plays positive roles in the catalysis for suc-Ala-Ala-Ala-pNA. The k_{cat} of these enzymes for the substrate are 4-7 times that of the wild-type enzyme. The replacement of Ser^{102} by histidine resulted in no activation of the catalysis of suc-Ala-Ala-Ala-pNA. In most cases, the k_{cat} values for suc-Ala-Ala-Ala-pNA increased, while the k_{cat} values for suc-Phe-Ala-Ala-pNA decreased. The introduction of a bulky side chain at Ser^{102} or Gly^{131} altered the P3-specificity of the enzyme.

Mutations at Ser^{102} or Gly^{131} also affected the K_m values. In every case, the K_m rose by replacement of the target residue. These results suggest that the introduced side chains should be situated on the edge of the substrate binding cleft, which might destabilize the Michaelis-complex in the proteolytic reaction, while the transition states for the cleavage of the Ala-peptide are stabilized.

Hydrolysis of the Standard Peptide—Another peptide, suc-Ala-Ala-Pro-Phe-pNA, a standard substrate that is often used for subtilisins assays (*1-13*), was used to analyze whether peptide length affects P3-specificity. The tetrapeptide substrate, suc-Ala-Ala-Pro-Phe-pNA, contains an alanine residue in the position of the P3 site. The kinetic parameters are shown in Table IV. All mutant enzymes, as well as the wild-type enzyme, hydrolyzed this substrate more efficiently than tripeptide substrates. The parameters of the mutant enzymes S102H and S102E were identical to those of the wild-type enzyme, as observed in the case of tri-alanine substrate. Results of tetrapeptide substrate were consistent with those of tripeptide substrates. These data show that the replacement of Ser^{102} by histidine has no effect on the recognition of alanine residues in the position of the P3 site. The replacement of Ser^{102} by lysine caused an activation of proteolytic activity. The k_{cat} of S102K was about 4.4-times higher than that of the wild-type enzyme, while every replacement of Gly^{131} resulted in decreasing the enzyme activity. In contrast to the results with the tripeptide substrate containing an alanine residue at the P3 site, replacement of Gly^{131} by histidine or lysine resulted in a decrease in the enzyme activity. We think these differences are attributable to the length of the peptide and to the existence of a proline residue at the P2 site of the tetrapeptide substrate.

The results suggested that the P3 specificities of the mutant proteins depend on substrate length. The introduction of acidic amino acid residues at Ser^{102} and Gly^{131} resulted in a decrease in the proteolytic activities in the case of the tripeptide substrate. These results may contribute to the electrostatic interaction between the acidic side chain of the S3 site and the succinyl group of the substrate in the position of the P4 site.

Comparison of Target Residues of Other Serine Proteases—The partial alignment of the amino acid sequences around the target S3 site of various serine proteases are summarized in Table II. The serine and the glycine residues are highly conserved. Our results indicate that the replacement of serine or glycine by other residues affects the P3-specificity, by excluding large P3-residues, that might restrict the narrow variation of the target peptide sequences to be recognized as substrates.

Serine and glycine are highly conserved in the proteases used as structural models, subtilisin BPN′, subtilisin Carlsberg, thermitase, and proteinase K. The P3-specificities of these enzymes are similar to that of wild-type aqualysin I; the S3 sites of these enzymes prefer large hydrophobic residues (*43-46*).

TABLE III. **Kinetic analysis of S3 site mutants *versus* substrates having variable P3 residues.**

Mutant	P3 residue					
	Ala			Phe		
	k_{cat}	K_m	k_{cat}/K_m	k_{cat}	K_m	k_{cat}/K_m
Wild type	1.2	1.1	1.1	11	0.044	250
S102H	1.6	2.1	0.76	0.11	0.24	0.45
S102K	8.5	10	0.83	2.9	0.15	20
S102E	0.5	3.8	0.13	0.11	0.34	0.33
G131H	7.3	7.1	1.0	1.1	0.52	2.1
G131K	5.4	2.3	2.4	1.5	0.51	3.0
G131D	2.4	2.1	1.1	ND	>1	13

Kinetic parameters for the hydrolyses of the tripeptide series suc-X-Ala-Ala-pNA, where X was Phe or Ala. 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, $mM^{-1} \cdot s^{-1}$. Standard errors were less than 22%. Parameters of the wild-type enzyme are from Tanaka *et al.* (39), in which assays were performed under the same conditions as this work. Parameters of the G131D protein for suc-Phe-Ala-Ala-pNA were not determined, because of the large K_m value over the solubility limit of the substrate. Only an apparant second-order rate constant, k_{cat}/K_m, was determined.

TABLE IV. **Hydrolysis of the standard peptide.**

Mutant	suc-Ala-Ala-Pro-Phe-pNA		
	k_{cat}	K_m	k_{cat}/K_m
Wild type	33	1.2	28
S102H	54	1.8	30
S102K	145	1.7	85
S102E	25	1.1	24
G131H	8.9	1.9	4.7
G131K	9.0	2.0	4.4
G131D	7.9	0.49	16

Kinetic parameters for the hydrolyses of the tetrapeptide suc-Ala-Ala-Pro-Phe-pNA. Assays were carried out 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, $mM^{-1} \cdot s^{-1}$. Standard errors were less than 7.9%. Parameters for the wild-type enzyme are from Tanaka *et al.* (39), in which assays were done under the same conditions as in this work.

Conclusions—We identified two residues, Ser^{102} and Gly^{131}, in aqualysin I that are located in the S3 site. The P3-specificity of the enzyme is altered by the replacement of these residues so as to build "walls" within the S3 site to form a pocket. The introduction of a large side chain at these positions tends to raise the catalytic efficiency for small residue, alanine, and to reduce the catalytic efficiency for a large residue, phenylalanine. The altered P3-specificities of Gly^{131}-derivatives were peptide-length dependent.

As all simulations described in this paper were done on the structures of subtilisin and its related enzymes, the strategies described here are applicable to designing the S3 site design of all subtilisin-related enzymes.

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