Restriction of substrate specificity of subtilisin E by introduction of a side chain into a conserved glycine residue

Hiroshi Takagi[a,*], Tadao Maeda[a], Iwao Ohtsu[a], Ying-Chieh Tsai[b], Shigeru Nakamori[a]

[a]*Department of Bioscience, Fukui Prefectural University, 4-1-1 Kenjojima, Matsuoka-cho, Yoshida-gun, Fukui 910-11, Japan*
[b]*Institute of Biochemistry, National Yang-Ming Medical College, Shih-Pai, Taipei, 11221, Taiwan, ROC*

Received 10 July 1996; revised version received 26 August 1996

Abstract Substitution of the conserved Gly^{127} for residues having a side chain markedly changed the substrate specificity of subtilisin E from *Bacillus subtilis*. The crystallographic findings suggested that Gly^{127} is responsible for accepting even the large P1 substrates, and the marked change of specificity was attributed to the introduction of a side chain in this position. To test this hypothesis, Gly^{127} was replaced with 3 non-charged amino acids, Ala, Ser and Val. When assayed with synthetic peptide substrates, all mutants purified from the periplasmic space in *Escherichia coli* showed a marked preference for small P1 substrate up to 150-fold relative to the wild-type. The kinetic data and molecular modeling analysis suggest that large hydrophobic P1 residues were unable to access the binding pocket due to steric hindrance.

Key words: Subtilisin; Substrate specificity; Site-directed mutagenesis

1. Introduction

Although many proteases with various substrate specificities exist in nature, one of the most important aims of protein engineering is to design and create new proteases with high preferences for specific substrates, which can be applied for site-specific proteolysis. Extensive studies on well-characterized enzymes have shown that substrate specificities can be modified by mutating the amino acid residues to which the substrate directly binds [1–3]. For instance, in view of its industrial applications in detergents and food processing, subtilisin produced by various *Bacillus* species has been extensively studied using site-directed mutagenesis [4,5]. From crystallographic analysis [6], the substrate binding pocket in subtilisins, which are characterized by broad specificities, is made up both of the main chain of Ser^{125}-Leu^{126}-Gly^{127} and the main and side chains of Ala^{152}-Ala^{153}-Gly^{154}, and Gly^{166} is located at the bottom of the pocket for the P1 substrate side chain and plays a critical role in determining their specificities. In subtilisins, the pocket is large and hydrophobic, which explains the broad specificity of this enzyme with a preference for aromatic or large non-polar P1 and P4 substrate residues [7]. Previous studies on subtilisin [7–10] have shown that the P1 specificity can be changed by substitution of amino acid residues to which a substrate binds directly. In fact, all 19 possible amino acid substitutions for Gly^{166} in subtilisin BPN′ have been tried by cassette mutagenesis, and the effects of hydrophobic interaction on substrate specificity have been systematically investigated [8]. On the other hand, Gly^{127} is absolutely conserved in the bacterial subtilisin family [11], and it contacts the P1, P3, and P4 substrate residues according to the X-ray coordinates for bound products in subtilisins BPN′ and Carlsberg [7]. Therefore, the residue at position 127 is thought to be an important site for determining the specificity of subtilisin, however, the possible contribution of side chains at this position to substrate binding is unknown.

Using an *Escherichia coli* expression system [12,13], we also analyzed the substrate specificity of *Bacillus subtilis* subtilisin E based on the structure of a new alkaline elastase produced by the alkalophilic *Bacillus* strain, which has very high elastolytic activity and indicated that deletion of the four amino acids, Ser^{161}-Thr^{162}-Ser^{163}-Thr^{164}, in the vicinity of the P1 binding pocket appeared to influence not only its substrate specificity but also its catalytic efficiency [14]. In the present study, we focused upon the conserved residue constituting P1 substrate binding pocket, and found that the substitution of Gly^{127} with Ala, Ser and Val showed high specificity towards the small P1 side chain, whereas the catalytic activity toward large P1 side chains and casein as the substrates for wild-type subtilisin was severely impaired.

2. Materials and methods

2.1. Materials

An *E. coli* strain JA221(*hsdM*$^+$ *trpE5 leuB6 lacY recA1/F′ laqI*^{q} *lac*$^+$ *pro*$^+$) [15], was used as a host. The isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible pIN-III-ompA vector [16] was used for the expression and secretion of mutant and wild-type subtilisin E. All enzymes for DNA manipulations were obtained from Takara Shuzo and used under conditions recommended by the supplier. Oligonucleotides were synthesized on a model 380A DNA synthesizer from Applied Biosystems using phosphoramidite chemistry [17] and purified by high-performance liquid chromatography. Synthetic peptide substrates were purchased from Bachem Feinchemikalien AG and Sigma. Casein (Hammarsten) was obtained from Merck.

2.2. Site-directed mutagenesis

Amino acid substitution was carried out according to the method of Inouye and Inouye [18] directly on the plasmids, and the mutations were confirmed by dideoxy-chain termination sequencing [19]. The amber (UAG) termination codon at position 127 was introduced with 5′-CATGAGCCTTT*A*G*GGACCTAC-3′ (asterisks show the locations of mismatches). To derive active mutants with various amino acid substitutions at position 127, a plasmid harboring the incomplete subtilisin gene introducing termination codon at position 127 was used as a template DNA for mutagenesis, and the following mixture of oligonucleotides was used as a primer:

5′-CATGAGCCTTGGGGGACCTAC-3′
CCC
AA
TT

The replacement of Gly^{127} by Ala, Ser and Val was performed with 5′-TGAGCCTTGC*CGGACCTAC-3′, 5′-TGAGCCTTA*GCGGACCTAC-3′, and 5′-TGAGCCTTGT*CGGACCTAC-3′, respectively. A plasmid harboring the wild-type sequence (pHI212) [20] was used as a template DNA for site-directed mutagenesis.

*Corresponding author. Fax: (81) (776) 61-6015.
E-mail: hiro@fpu.ac.jp

PII S0014-5793(96)01014-9

2.3. Isolation of mutant subtilisins containing the caseinolytic activity

First, various amino acid substitutions of subtilisin gene at position 127 were made by site-directed mutagenesis as described above. The DNA mixture containing the heteroduplex derived from the primer and the template was transformed into *E. coli* strain JA221 on M9 [21] agar plates supplemented with 0.2% casamino acids, 0.4% glucose, 0.02% $MgSO_4$, 0.05 mg/ml tryptophan, 0.5 μg/ml vitamin B_1, and 50 μg/ml ampicillin containing 2% casein [22]. After incubation of the plates at 37°C overnight, they were then incubated at room temperature. Transformants producing active subtilisins were then selected on the plate by detecting halo formation, which indicates partial hydrolysis of casein. No halo was formed by *E. coli* harboring pHT127TAG (termination codon at position 127) as a template DNA, because of the incomplete tertiary structure of subtilisin. Thus, colonies producing the active subtilisin can be selected from those of the parent cell. After re-transformation, the plasmid DNA was purified from each mutant and sequenced to identify the amino acid substitution.

2.4. Expression and purification of wild-type and mutant subtilisins E

Wild-type and mutant subtilisin E genes were expressed in *E. coli* strain JA221, cultivated at 37°C in M9 medium supplemented with 2% casamino acids, 0.4% glucose, 0.02% $MgSO_4$, 0.05 mg/ml tryptophan, 0.5 μg/ml vitamin B_1, 1 mM $CaCl_2$, and 50 μg/ml ampicillin. When A_{570} reached 0.4, the cultivation temperature was reduced to 25°C and IPTG was added to the culture medium to a final concentration of 1 mM for induction of gene expression. After cultivation for 4 h at 25°C, the cells were harvested by centrifugation. The periplasmic fraction was prepared according to the method of Koshland and Botstein [23]. To purify the wild-type and mutant subtilisins E, the periplasmic fraction of induced cells was first applied to a cation-ion exchange CM-Sepharose Fast Flow column (Pharmacia), which was equilibrated with 10 mM sodium phosphate buffer (pH 6.2). The subtilisin was eluted with 80 mM NaCl, and subsequently applied to a cation-ion exchange Mono S-Sepharose Fast Flow column (Pharmacia). The protein peak eluted with a 0–160 mM NaCl gradient was collected. Enzyme concentration were determined spectrophotometrically. The eluted active fraction showed a single protein band of subtilisin E upon SDS-polyacrylamide gel electrophoresis.

2.5. Assay of enzymatic activity

For synthetic peptide substrates, assays were performed as described previously [20]. The amount of *p*-nitroaniline released was measured by the absorbance at 410 nm and activity was calculated as units/mg protein. One unit is defined as the activity releasing 1 μmol of *p*-nitroaniline/min. Caseinolytic activity was assayed by the method of Hagihara et al. [24]. One unit is defined as the quantity required to increase the absorbance at 660 nm by the equivalent of 1 μg of tyrosine/min at 37°C.

3. Results

3.1. Design of mutation

In the family of subtilisin-like serine proteinases, the two sides of the P1 cleft are formed by the backbone segments 125–128 and 152–155, while the segment 166–169 forms the bottom of the cleft. In particular, the Gly residue at position 127 can contact the P1, P3, and P4 substrate residues [7], and is absolutely conserved among the family (Fig. 1). Although the three-dimensional structure of subtilisin E at 2.0 Å resolution was recently determined [25], we predicted the structure based on the crystallographic data for subtilisin BPN′, which is 86% identical in amino acid sequences with subtilisin E. Molecular modeling showed that Gly^{127} is located on one side of the P1 substrate binding cleft, and seems to be responsible for the broad P1 specificity of this enzyme. We postulated that the introduction of side chains into this position in subtilisin E might affect the conformation of the P1 cleft, and thereby, cause alteration of the substrate specificity. To examine this possibility, we chose the residue at position 127 for site-directed mutagenesis.

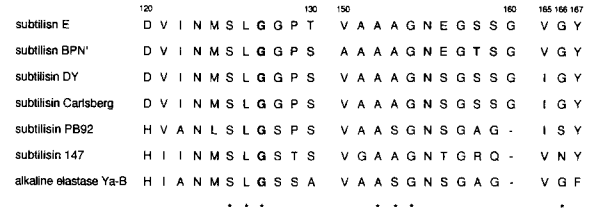

Fig. 1. Alignment of amino acid sequences of substrate binding pocket domains of *B. subtilis* 168 subtilisin E, *B. amyloliquefaciens* subtilisin BPN′, *B. subtilis* DY subtilisin DY, *B. licheniformis* subtilisin Carlsberg, *B. alcalophilus* PB92 subtilisin PB92, *B. lentis* subtilisin 147, and the alkalophilic *Bacillus* sp. Ya-B alkaline elastase Ya-B. All the sequences shown are cited from [11]. The numbering above the amino acid sequenses refers to suntilisin E. Asterisks indicate the residues involved in the substrate binding pocket, and Gly^{127} is shown in bold. Horizontal line indicate that absence of a corresponding amino acid residues at this position. From the crystallographic analysis of subtilisin BPN′ [6], the substrate binding pocket in subtilisins, which are characterized by broad specificities, is made up of both the main chain of residues 125-126-127 and the main and side chains of residues 152-153-154, and residue 166 is located at the bottom of the pocket.

3.2. Isolation of suppressor mutant subtilisins by site-directed mutagenesis

The UGA termination codon at position 127 in subtilisin E was first introduced by site-directed mutagenesis, and the cells carrying the mutated gene did not form any halo on a casein agar plate due to the structural destruction. We then attempted to isolate suppressors from the incomplete subtilisin by introducing various amino acid residues in place of the termination codon as described in Section 2. From approx. 10^5 colonies, several halo-forming colonies were isolated on a casein agar plate. Although all subtilisin genes from these positive colonies were sequenced, they were all wild-type subtilisin genes having Gly at position 127. In this study, none of the mutant subtilisins showing caseinolytic activity were obtained. This suggests that Gly at position 127 plays an important role in the expression of caseinolytic activity.

3.3. Construction and expression of mutant enzymes

To introduce the side chains with different volumes in this residue, three mutant plasmids, pTMG127A, pTMG127S, and pTMG127V, were constructed to replace Gly^{127} with Ala, Ser, and Val, respectively. Wild-type and mutant subtilisin E genes were expressed in *E. coli*. We first examined their abilities to form halos on a casein agar plate. However, none of the cells carrying the mutations formed clearly discernible halos even after 7 days incubation at 25°C. This suggested that the substitution of Gly^{127} with Ala, Ser, and Val resulted in decreased proteinase activity compared to the wild-type enzyme.

3.4. Catalytic properties of mutant enzymes

After passage through two ion-exchange columns, all mutant enzymes were purified from the periplasmic fraction to give a single band upon SDS-polyacrylamide gel electrophoresis, and their enzymatic properties were investigated. Wild-type subtilisin E purified from *E. coli* harboring plasmid pHI212 was examined as a control. Purified enzymes were assayed with *N*-succinyl-L-Ala-L-Ala-L-Pro-L-X-*p*-nitroanilide (AAPX; X=Phe (F), Ala (A), Met (M), Leu (L), and Lys (K)). AAPF has been used as an authentic substrate for subtilisin. AAPA has been regarded as a typical P1 residue having

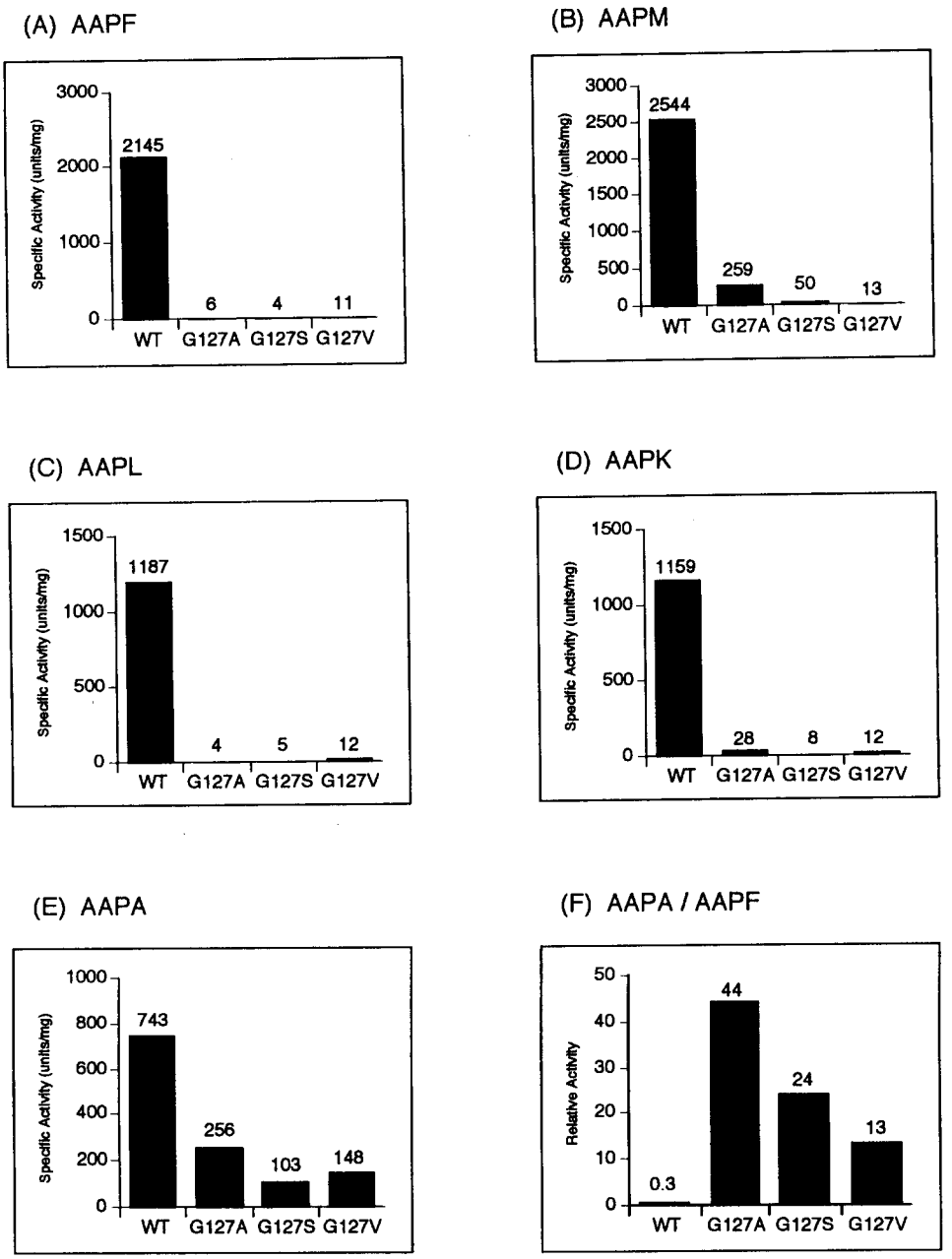

Fig. 2. Hydrolysis of peptide substrates by the wild-type and the various mutant subtilisins E. Assays were performed in 50 mM Tris-HCl (pH 8.5) and 1 mM $CaCl_2$ at 37°C using AAPX (succinyl-Ala-Ala-Pro-X-*p*-nitroanilide; X is Phe (F), Met (M), Leu (L), Lys (K), and Ala (A)). The wild-type and mutants $Gly^{127} \rightarrow$ Ala, Ser, and Val enzymes are shown as WT, G127A, G127S, and G127V, respectively. Variations in the values were below 5%.

a small side chain. Fig. 2 shows the specific activity toward five peptide substrates, and the relative activity of AAPA/AAPF of the enzymes. The specific activity toward AAPF, AAPK, and AAPL as substrates for subtilisin was severely impaired when the side chain was introduced at position 127, in particular, all the mutants had less than 1% of the wild-type enzymatic activity for AAPF. Consequently, in the case of casein as a natural substrate, the hydrolyzing activities of the mutants were greatly diminished relative to that of the wild-type enzyme (less than 100 units/mg for the mutants vs. 896 units/mg for the wild-type). However, the activity of the mutants for AAPA remained approx. 15–35% of that of the wild-type enzyme. It is worth noting that all mutants showed a marked increase in the AAPA/AAPF ratio ranging from 40- to 150-fold.

Kinetic constants k_{cat} and K_m were determined from initial rate measurements for hydrolysis of AAPF, AAPM, and AAPA. As shown in Table 1, it was impossible to obtain the individual kinetic parameters k_{cat} and K_m of the mutants for AAPF (except for the Val mutant) which reflects its catalytic efficiency, due to the marked increase in K_m for this substrate and limited substrate solubility, which indicates little affinity of the enzyme for AAPF. On the other hand, the Gly^{127} mutations resulted in only a 2-fold increase in K_m values for AAPA with respect to wild-type enzyme. Therefore, the k_{cat}/K_m ratio of the mutants remains approx. 7–25% of

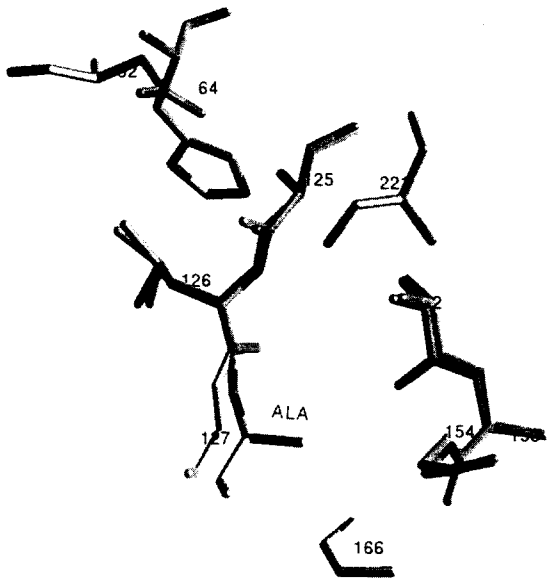

Fig. 3. Computer modeling of the P1 substrate binding pocket in the predicted structure of the α-carbon backbone structure of subtilisins E. Wild-type (Gly^{127}) and Gly^{127}Ala mutant subtilisins E are drawn as the predicted structure using program Insight II, and Homology (MSI softwares).

that of the wild-type enzyme, although the k_{cat} value decreased significantly. These findings suggest that after introduction of the side chain into position 127, only the small P1 substrate could be accepted at the binding pocket, not the large ones, leading to the marked change in substrate preference. In addition, it might cause the structural change around the active site that showed considerably lower specific activity toward the substrates for subtilisin.

Computational modeling was conducted to predict the structure around the substrate-binding pocket in the mutant enzymes. A part of them (Ala substitution) is shown in Fig. 3. The modeling of the mutants suggested that the steric hindrance due to the introduction of Ala, Ser, or Val side chain at position 127 might occur at the entrance of the cleft, which is formed by the segments Ser^{125}-Leu^{126}-Gly^{127} and Ala^{152}-Ala^{153}-Gly^{154}. The steric hindrance can be explained on the basis of the distances between the α-carbon backbones of the cleft. The values in the mutants were shorter than those of wild-type (Table 2), and the side chain molecular volumes of Ala, Ser, and Val (15.3, 21.7, and 36.5 Å, respectively) are considerably larger compared to Gly. Such a difference around the substrate binding pocket might be responsible for the different specificities of the enzymes.

4. Discussion

Subtilisin has broad specificity and contains a large hydrophobic substrate binding pocket. One of the most important aims of protein engineering in subtilisin is to analyze the broad P1 substrate specificity in order to restrict substrate preference. Previous protein engineering studies of subtilisin [7–10] have shown that substrate specificity can be changed by replacement of amino acid residues to which a substrate binds directly. A conserved Gly at position 166, located at the bottom of the substrate binding pocket, was replaced by nonionic amino acids [8]. In general, the catalytic efficiency toward small hydrophobic substrates was increased by hydrophobic substitutions at position 166 in the binding pocket. Surpassing the optimal binding volume of the pocket, by introducing either the substrate side chain or the side chain at position 166, caused a significant decrease in catalytic efficiency due to steric hindrance. Also, a general change in substrate specificity resulting from charged amino acid substitutions at residue 156 and 166 in the P1 binding site led to electrostatic effects [9]. This was supported by data showing the catalytic efficiency toward complementary charged P1 substrates and decrease toward charged P1 substrates. Furthermore, Wells et al. [7] reported the feasibility of recruitment of substrate specificity properties from subtilisin BPN′ into a related species (Carlsberg) by three substitutions at positions 156, 169, and 217 that are within the van der Waals contact of the substrate. However, no attention has been given to Gly^{127} as a possible site of engineering, although position 127 is thought to be one of the P1 substrate binding sites [7]. Position 127 is highly occupied by Gly in the subtilisin family (Fig. 1). The role of amino acid 127 is supposedly to interact with the P1 substrate and to constitute the one side of the substrate binding pocket with Ser^{125} and Leu^{126}. We postulated that the introduction of a side chain to position 127 by site-directed mutagenesis would greatly affect the substrate specificity,

Table 1
Kinetic constants of the wild-type and the various mutant subtilisins E

Substrate	Enzyme	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
AAPF	Wild-type	17.6 ± 0.52	1.85 ± 0.05	9.48 ± 0.04
	G127A	N.D.	N.D.	N.D.
	G127S	N.D.	N.D.	N.D.
	G127V	0.55 ± 0.10	2.63 ± 0.11	0.21 ± 0.04
AAPM	Wild-type	19.7 ± 0.48	1.03 ± 0.02	19.1 ± 0.17
	G127A	2.75 ± 0.08	3.23 ± 0.08	0.85 ± 0.01
	G127S	0.57 ± 0.03	3.23 ± 0.11	0.18 ± 0.01
	G127V	0.36 ± 0.02	3.33 ± 0.10	0.11 ± 0.01
AAPA	Wild-type	2.89 ± 0.60	0.83 ± 0.03	3.44 ± 0.61
	G127A	1.41 ± 0.07	1.79 ± 0.14	0.79 ± 0.04
	G127S	0.44 ± 0.01	1.79 ± 0.05	0.25 ± 0.01
	G127V	0.75 ± 0.01	1.72 ± 0.07	0.44 ± 0.01

Assays were performed in 50 mM Tris-HCl (pH 8.5) and 1 mM $CaCl_2$ at 37°C using AAPF (succinyl-Ala-Ala-Pro-Phe-*p*-nitroaniliide), AAPM (succinyl-Ala-Ala-Pro-Phe-*p*-nitroaniliide), and AAPA (succinyl-Ala-Ala-Pro-Ala-*p*-nitroaniliide). N.D., not determined since individual k_{cat} and K_m values could not be obtained due to limited substrate solubility.

Table 2
Predicted distances between α-carbon backbones of the P1 substrate binding pocket of the wild-type and the various mutant subtilisins E

Residue	α-Carbon distances (Å)			
	Wild (Gly^{127})	Ala^{127}	Ser^{127}	Val^{127}
Ala^{152}	7.06	6.30	6.41	6.56
Ala^{153}	7.76	6.91	7.02	7.23
Gly^{154}	6.40	5.68	5.93	6.39

The distances are given between residue 127 and segment Ala^{152}-Ala^{153}-Gly^{154} forming the P1 binding pocket using program Insight II and Homology (MSI softwares).

while side chains of Ser^{125} and Leu^{126} extend outside of the pocket.

To isolate suppressor mutants from the incomplete subtilisin gene introducing termination codon at position 127, we tried all 20 amino acid substitutions (site-saturation) using the mixture of oligonucleotides. As an approach to structure-function studies, site-saturation is considered to be most appropriate, because of uncertainties in predicting which amino acid would be an optimal residue. Despite the fact that the suppressor mutants could be screened by detecting halo formation due to subtilisin activity, we were not able to isolate any mutants showing high proteolytic activity, except for wild-type. Similarly, note that cells expressing the Ala, Ser, and Val mutant subtilisins did not develop clear halos (data not shown). Judging from these observations, it was concluded that Gly residue at position 127 is indispensable for the catalytic activity as subtilisin.

The most notable finding of the present study is the change of the P1 substrate specificity by introduction of side chains to position 127 in subtilisin. Our findings strongly suggest that the volume of side chain equivalent to one methyl group would be enough not to allow the large P1 substrate side chains to be accepted, since Ala substitution at position 127 caused a marked reduction in activity with AAPF, AAPL, and AAPK as the favored substrates for subtilisin. The mutant enzyme with the substitution for Val showed hydrolyzing activity only for Ala as P1 substrate. The mutant enzymes might be very useful in protein chemistry, and industry, even though their specific activity should be enhanced. The fact that the k_{cat} and K_m values for AAPF in Gly^{127}Ala and Gly^{127}Ser mutants were unable to be determined support the possibility of the loss of binding toward large P1 substrate. Indeed, when the α-carbon distances between residues forming the cleft were predicted by molecular modeling (Table 2), steric hindrance in the mutants would obviously occur. In particular, it was speculated that Ala substitution at position 127 led to the greatest hindrance effect caused by the shortest distance. Similarly, the crystal structures of mammalian serine proteases (trypsin and chymotrypsin) showed a correlation between the P1 specificity of the enzyme and the conformation of the polypeptide backbone at Gly^{216}, which is the analog of Gly^{127} in these enzymes [26]. In mammalian elastases, Val^{216} plays a dual role both by providing a hydrophobic pocket for the P1 residue and by forming the main chain hydrogen bonds at position P3 [27]. Therefore, conversion of either trypsin or chymotrypsin to an elastase-like protease is predicted to require mutations sufficient to reorient the main chain of position 216 to an elastase-like conformation [26].

The question arises as to why the amino acid replacement at position 127 causes a significant decrease in catalytic efficiency, mainly caused by an alteration in k_{cat} and not in K_m, whereas the substrate having the Ala residue in P1 was hydrolyzed effectively. The reason for the decrease in the catalytic efficiency of the mutants relative to the wild-type enzyme remains unknown.

A similar finding was obtained for the cysteine protease cathepsin B exhibiting broad P1 specificity [28]. These findings indicate that the specificity changes resulting from P1 pocket mutations are partly the result of large disruption in enzyme structure. An X-ray crystallographic study by Kraut et al. [29] revealed that there is a hydrogen bond between Asp^{32} and His^{64}, but there is none between the catalytic side chains of Ser^{221} and His^{64} being constituents of the catalytic triad in subtilisin. Although the mechanism is not elucidated by the structural prediction around the substrate-binding pocket in the mutant enzymes, it seems that with the Ala, Ser, and Val substitutions at position 127, a slight change of distance between the catalytic triad may occur, diminishing proton transfer. On the other hand, it may appear surprising that a conserved Gly^{166} can be substituted to maintain activities almost equal to the wild-type enzyme with narrower specificity [8]. Further research on the active site of the mutants is currently in progress.

In conclusion, we found that it is possible to create a novel protease having highly limited substrate specificity from the wild-type enzyme by replacing the conserved residue related to the substrate binding pocket.

Acknowledgements: We thank Ryoka Systems Inc. (Chiba, Japan) for computational modeling, and Mr. Yukihiro Yabuta for the preparation of figures.

References

[1] Craik, C.S., Largman, C., Fletcher, T., Roczniak, S., Barr, P.J., Fletterick, R. and Rutter, W.J. (1985) Science 228, 291–297.
[2] Wilks, H.M., Hart, K.W., Feeney, R., Dunn, C.R., Muirhead, H., Chia, W.N., Barstow, D.A., Atkinson, T., Clarke, A.R. and Holbrook, J.J. (1988) Science 242, 1541–1544.
[3] Bone, R., Silen, J.L. and Agard, D.A. (1989) Nature 339, 191–195.
[4] Wells, J.A. and Estell, D.A. (1988) Trends Biochem. Sci. 13, 291–297.
[5] Takagi, H. (1993) Int. J. Biochem. 25, 307–312.
[6] Wright, C.S., Alden, R.A. and Kraut, J. (1969) Nature 221, 235–242.
[7] Wells, J.A., Cunningham, B.C., Graycar, T.P. and Estell, D.A. (1987) Proc. Natl. Acad. Sci. USA 84, 5167–5171.
[8] 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.
[9] Wells, J.A., Powers, D.B., Bott, R.R., Graycar, T.P. and Estell, D.A. (1987) Proc. Natl. Acad. Sci. USA 84, 1219–1223.
[10] Carter, P., Nilsson, B., Burnier, J.P., Burdick, D. and Wells, J.A. (1989) Proteins: Struct. Funct. Genet. 6, 240–248.
[11] Siezen, R.J., De Vos, W.M., Leunissen, J.A.M. and Dijkstra, B.W. (1991) Protein Eng. 7, 719–737.
[12] Ikemura, H., Takagi, H. and Inouye, M. (1987) J. Biol. Chem. 262, 7859–7864.
[13] Takagi, H., Morinaga, Y., Tsuchiya, M., Ikemura, H. and Inouye, M. (1988) Bio/Technology 6, 948–950.
[14] Takagi, H., Arafuka, S., Inouoye, M. and Yamasaki, M. (1992) J. Biochem. 111, 584–588.
[15] Nakamura, K., Masui, Y. and Inouye, M. (1982) J. Mol. Appl. Genet. 1, 289–299.
[16] Ghyayeb, J., Kimura, H., Takahara, M., Hsiung, H., Masui, Y. and Inouye, M. (1984) EMBO J. 3, 2437–2442.
[17] Shinha, N.D., Biernat, J., McManus, J. and Koster, H. (1984) Nucleic Acids Res. 12, 4539–4557.

[18] Inouye, M. and Inouye, S. (1987) in: Synthesis of DNA, RNA and Their Application (Narang, S. ed.) pp. 181–206, Academic Press, New York.
[19] Sanger, F., Coulson, A.R., Barrell, B.G., Smith, A.J.H. and Roe, B.A. (1980) J. Mol. Biol. 143, 161–178.
[20] Takagi, H., Morinaga, Y., Ikemura, H. and Inouye, M. (1988) J. Biol. Chem. 263, 19592–19596.
[21] Miller, J.H. (1972) in: Experiments in Molecular Genetics (Jeffrey, H.M. ed.) pp. 431–432, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[22] Takagi, H., Morinaga, Y., Ikemura, H. and Inouye, M. (1989) J. Biochem. 105, 953–956.
[23] Koshland, D. and Botstein, D. (1980) Cell 20, 749–760.
[24] Hagihara, B., Matsubara, H., Nakai, M. and Okunuki, K. (1958) J. Biochem. 45, 185–194.
[25] Chu, N.-M., Chao, Y. and Bi, R.-C. (1995) Protein Eng. 8, 211–215.
[26] Perona, J.J., Hedstrom, L., Rutter, W.J. and Fletterick, R.J. (1995) Biochemistry 34, 1489–1499.
[27] Navia, M.A., McKeever, B.M., Springer, J.P., Lin, T.-Y., Williams, H.R., Fluder, E.M., Dorn, C.P. and Hoogsteen, K. (1989) Proc. Natl. Acad. Sci. USA 86, 7–11.
[28] Fox, T., Mason, P., Storer, A.C. and Mort, J.S. (1995) Protein Eng. 8, 53–57.
[29] Matthews, D.A., Alden, R.A., Birktoft, J.J., Freer, S.T. and Kraut, J. (1977) J. Biol. Chem. 252, 8875–8883.