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Improved Autoprocessing Efficiency of Mutant Subtilisins E with Altered Specificity by Engineering of the Pro-Region[1]

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Modification of substrate specificity of an autoprocessing enzyme is accompanied by a risk of significant failure of self-cleavage of the pro-region essential for activation. Therefore, to enhance processing, we engineered the pro-region of mutant subtilisins E of *Bacillus subtilis* with altered substrate specificity. A high-activity mutant subtilisin E with Ile31Leu replacement (I31L) as well as the wild-type enzyme show poor recognition of acid residues as the P1 substrate. To increase the P1 substrate preference for acid residues, Glu156Gln and Gly166Lys/Arg substitutions were introduced into the I31L gene based upon a report on subtilisin BPN' [Wells *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84, 1219–1223]. The apparent P1 specificity of four mutants (E156Q/G166K, E156Q/G166R, G166K, and G166R) was extended to acid residues, but the halo-forming activity of *Escherichia coli* expressing the mutant genes on skim milk-containing plates was significantly decreased due to the lower autoprocessing efficiency. A marked increase in active enzyme production occurred when Tyr(–1) in the pro-region of these mutants was then replaced by Asp or Glu. Five mutants with Glu(–2)Ala/Val/Gly or Tyr(–1)Cys/Ser substitution showing enhanced halo-forming activity were further isolated by PCR random mutagenesis in the pro-region of the E156Q/G166K mutant. These results indicated that introduction of an optimum arrangement at the cleavage site in the pro-region is an effective method for obtaining a higher yield of active enzymes.

Key words: autoprocessing enzyme, pro-region, protein engineering, substrate specificity, subtilisin E.

Substrate specificity is one of the most characteristic features of proteases. Accumulation of three-dimensional structural data and advances in protein engineering have allowed the substrate specificity of proteases successfully to be modified (*1–4*). However, in the case of autoprocessing proteases, such modifications may cause significant failure of cleavage of the pro-regions, which is necessary for conversion of the inactive pro-enzyme into the active mature form (*5–7*).

Using an *Escherichia coli* expression system, we engineered subtilisin E (SBT) derived from *Bacillus subtilis* strain 168 to change the substrate specificity and enhance the stability at high temperature or in organic solvents (*8–11*). SBT needs the pro-region for appropriate folding as an intramolecular chaperone when it is newly synthesized *in vivo*, and then the pro-region must be cleaved off by the enzyme itself for activation (*12–15*). In this study, we examined whether modification in the pro-region of mutant SBTs with altered substrate specificity was an effective method for improvement of the decreased processing efficiency caused by the specificity change.

SBT hardly recognizes acid residues as P1[3] substrates. Because Gly166 and Glu156 residues of SBT constitute the P1 substrate-binding pocket and Wells *et al.* demonstrated that E156Q- and G166K/R-substituted mutants of subtilisin BPN', a subtilisin family protease, showed increased preference for Glu residue as P1 substrate (*16, 17*), we replaced Glu156 and Gly166 of SBT with Gln and Lys/Arg residues, respectively, using the expression system in *E. coli*. The apparent P1 preference of the resultant mutants prepared from the periplasmic space of *E. coli* was extended to acid residues, while the amounts of mature enzymes in the periplasm were markedly reduced. Then, we investigated whether the processing efficiency could be improved by site-directed and random mutagenesis in the pro-region of these mutants.

MATERIALS AND METHODS

Materials—An *E. coli* strain JM109 *(recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 Δ(lac-proAB)/F' [traD36 proAB⁺ lacI^q lacZΔM15])* and *B. subtilis* three proteases-deficient strain DB403 *(trpC2 aprE⁻ eprE⁻ nprE⁻)*, gifts from Dr. R.H. Doi, were used as host cells for the production of SBTs. An *E. coli* strain C600 *(recA⁺ F⁻ thi-1 thr-1 leuB6 lacY1 tonA21 supE44 λ⁻)* was used for construction of expression plasmids for *B. subtilis*. The isopropyl-β-D-

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[3] The nomenclature employed here for the amino acid residues in substrates is NH_2-P*n*, ..., P2, P1, P1', P2', ..., P*n*'-COOH, where P1-P1' denotes the hydrolyzed bond.

Abbreviations: AAPX, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-X-*p*-nitroanilide; CBB, Coomassie Brilliant Blue; IPTG, isopropyl-β-D-thiogalactopyranoside; nt, nucleotide; PCR, polymerase chain reaction; PVDF, polyvinylidene difluoride; SBT, subtilisin E; PAGE, polyacrylamide gel electrophoresis.

thiogalactopyranoside (IPTG)-inducible pIN-III-ompA vector (*12*) and the shuttle-vector pHY300PLK (Takara Shuzo, Kyoto) were used for the expression and secretion of SBTs in *E. coli* and *B. subtilis*, respectively. Synthetic peptide substrates, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-X-*p*-nitroanilide (AAPX; X = Asp (D), Glu (E), Lys (K), and Phe (F)), were purchased from Bachem AG (Bubendorf, Switzerland) or Sigma (St. Louis, USA).

Site-Directed Mutagenesis—Site-directed mutagenesis was performed essentially according to the previous paper (*9*). The plasmid pHT212L (8.7 kb) (*11, 18*) containing I31L mutant (L31) gene inserted into pIN-III-ompA (7.5 kb) was used as the primary template for site-directed mutagenesis. The first polymerase chain reaction (PCR) was performed using GeneAmp™ PCR System 2400 (Perkin-Elmer Corporation, Applied Biosystems, Foster City, CA) with two sets of primers separately, *i.e.*, an upstream forward primer together with a mutation primer (–), and a mutation primer (+) together with a downstream reverse primer. After the excess primers had been removed from the first PCR products by a SUPREC™-02 spin column (Takara Shuzo), the second PCR was performed with the upstream and downstream primers on the mixture of the equimolar first PCR products. As for the upstream and downstream primers, P35(+) (5′-ATCGACAGCGGAATTGAC-3′) and P240(–) (5′-GTCGGGTGCTTAGAAAGA-3′) were used respectively to replace both Glu156 and Gly166 residues, whereas P-75(+) (5′-CTGCCGGAAAAAGCAGT-3′) and P60(–) (5′-AACTGCCGTCCTGGTAT-3′) were used respectively to replace Tyr(–1). The mutation primers used were as follows: Y(–1)D/E(+), 5′-ACATGAAGA(A/T)GCGCAAT-CT-3′; Y(–1)D/E(–), 5′-AGATTGCGC(A/T)TCTTCATGT-3′; E156Q(+), 5′-AAACCAAGGTTCATCCGGA-3′; E156Q(–), 5′-TCCGGATGAACCTTGGTTT-3′; G166K/R(+), 5′-GCAC-AGTCA(A/G)ATACCCTG-3′; G166K/R(–), 5′-CAGGGTAT-(C/T)TGACTGTGC-3′ (underlines indicate mismatches and "(X/Y)" indicates mixed nt of X and Y). The amplified fragment containing E156Q or G166K/R mutation was ligated to the *Hin*dIII- and *Nco*I-digested 8.1-kb fragment of pHT212L, whereas the fragment containing Y(–1)D/E was ligated to the *Not*I- and *Hin*dIII-digested 8.4-kb fragment of pHT212L. Double- and triple-mutants were obtained by the combination of the site-directed mutagenesis and ligation of the mutagenic fragments. To introduce the Y(–1)E replacement into AQK, HAE(+) (5′-GCACATGCAGAAG-CGCAATCT-3′) and HAE(–) (5′-AGATTGCGCTTCTGCAT-GTGC-3′) were used for the mutation primers and plasmid pHT212AQK was used for the template. The point mutations were confirmed on a ABI PRISM™ 377 DNA Sequencer (Applied Biosystems) using ThermoSequenase™ Dye Terminator Cycle Sequencing Kit (Amersham-Pharmacia Biotech, Tokyo).

Halo-Forming Assay—To examine the change in proteolytic activity easily, *E. coli* transformed with a plasmid expressing a mutant SBT was cultured on a skim milk-containing plate as described previously (*8*). Because lower temperature was suitable for prepro-SBT synthesized in *E. coli* cells to be efficiently secreted, folded and auto-processed, cells expressing SBT would not made halo at 37°C (*12*). Therefore, after a colony of *E. coli* was developed by incubation at 37°C for 20 h, the plate was transferred to 23°C and further incubated for 20 h unless otherwise indicated. Halo-forming activity was estimated by the diameter ratio of halo to colony.

Production and Purification of Mutant SBTs—Mutant SBTs were expressed in *E. coli* strain JM109 induced by 1 mM IPTG as described in the previous paper (*10*). Because the vector plasmid, pIN-III-ompA, had the signal sequence for ompA, the mutant enzymes would be secreted into the periplasmic space after being synthesized in *E. coli* cells. Hence, the periplasmic fraction was prepared by the method of Koshland and Botstein (*19*) and used for enzymatic reactions and in analysis by SDS–polyacrylamide gel electrophoresis (SDS-PAGE). Protein concentration was measured by Protein Assay kit (Nippon Bio-Rad Laboratories, Tokyo) using subtilisin BPN′ (Sigma) as a standard.

To construct a plasmid expressing a mutant SBT with altered specificity in *B. subtilis*, an *Nde*I- and *Bam*HI-digested fragment of pIOE (*11*) carrying a wild-type SBT gene inserted in a pHY300PLK was exchanged with that of the mutant. The nucleotide sequence was confirmed by DNA sequencing and the plasmid was introduced into *B. subtilis* DB403 by electroporation (*20*). Recombinant *B. subtilis* cells were grown at 37°C for 24 h in LB medium containing 20 μg/ml tetracycline. An ammonium sulfate precipitate (70% saturation) of the culture supernatant was applied onto a CM-Sepharose® Fast Flow column (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 10 mM sodium phosphate buffer (pH 6.2). The mutant SBT was eluted with 10 mM phosphate buffer (pH 6.2) containing 80–140 mM NaCl in a stepwise gradient, and the enzymatic activity of individual fractions was monitored by hydrolysis of AAPF. The active fractions containing a single protein band, and which exhibited the same mobility as standard wild-type SBT in SDS-PAGE, were collected as purified fractions.

Determination of N-Terminal Amino Acids—To investigate whether mutant pro-SBTs with altered specificity were processed on the C-terminal side of position –1, N-terminal amino acid sequences of the purified enzymes prepared from the culture supernatants of L31-, EQK-, and EQR-expressing *B. subtilis* cells were determined by Edman degradation using a Model 476A Protein Sequencer (Applied Biosystems).

Assay of Enzymatic Activity—Hydrolytic activity for synthetic peptide substrates, *i.e.*, AAPD, AAPE, AAPK, and AAPF, was investigated according to the previous paper (*18*). The enzyme reaction was performed in 50 mM Tris-HCl (pH 8.5) and 1 mM $CaCl_2$ at 37°C. The amount of released *p*-nitroaniline was determined by measuring the absorbance at 410 nm with a Beckman Spectrophotometer DU640 (Beckman Instruments, Fullerton, CA). The specific activities of periplasmic enzymes prepared from *E. coli* cells and purified mutant SBTs were calculated as units/mg-total protein. One unit is defined as the activity releasing 1 nmol of *p*-nitroaniline per min. The kinetic values of the hydrolysis with a purified mutant SBT were determined from the initial rates of the reaction.

SDS-PAGE and Immunoblotting—SDS-PAGE was performed according to the method of Laemmli (*21*). The periplasmic enzymes prepared from *E. coli* were separated by SDS-PAGE and either stained with Coomassie Brilliant Blue (CBB) R-250 or electrophoretically transferred onto polyvinylidene difluoride (PVDF) membrane according to the method of Towbin *et al.* (*22*). Wild-type and mutant SBTs on the membrane were detected by immunostaining

using the anti-mature SBT polyclonal antibody (*9*) and the Vectastain® ABC kit (Vector Laboratories, Burlingame, CA).

Random Mutagenesis in the Pro-Region of SBT—To introduce random mutations, a 610-bp fragment containing the pro-region (231 bp) of SBT was amplified by error-prone PCR using the pHT212QK plasmid carrying the QK mutant gene inserted into pIN-III-ompA as a template. Two oligonucleotides, lac-po (5′-GCTTCCGGCTCGTATAA-3′) and P60(–), of which sequences were located 423 nt upstream and 171 nt downstream of the 5′-end of the mature sequence in the pHT212QK, respectively, were used as primers. A 20-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.2 mM dATP, 1 mM dGTP, 1 mM dCTP, 1 mM dTTP, 0.05 mM $MnCl_2$, 2.5 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10% dimethylsulfoxide, 10 pmol of the each primer, 5 ng of template DNA, and 1 U of *rTaq* DNA polymerase (Toyobo Biochemicals, Osaka) was subjected to PCR for 25 cycles consisting of 96°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The mutagenic products were digested at the *Xba*I- and *Hin*dIII-restriction sites, located 332 and 144 nt upstream and downstream of the 5′-end of the mature sequence, respectively, and ligated to the *Xba*I- and *Hin*dIII-digested 8.2-kb fragment of pHT212QK. *E. coli* strain JM109 was transformed with the ligated plasmids and screened by halo-forming assay, and colonies exhibiting a larger halo than QK-expressing *E. coli* were isolated. The efficiency of introduction of mutation was estimated to be more than about 2.5% from the emergence probability of mutant colonies that no longer made halos even after 48 h incubation at 23°C. The plasmid DNA was prepared from the each clone and subjected to retransformation of *E. coli* to confirm that the enhanced halo-forming activity was owing to the plasmid itself.

To introduce into L31 the mutated pro-region obtained by the random mutagenesis, the *Xba*I- and *Hin*dIII-digested 0.5-kb fragment of L31 containing both pre- and pro-regions was exchanged with that of the random mutants. To introduce the E(–2)G replacement into QK or L31, the *Not*I- and *Hin*dIII-digested 0.2-kb fragment including only the pro-region of QK or L31 was exchanged with that of LGQK, yielding GQK and G(–2).

TABLE I. **Mutant SBTs obtained by site-directed mutagenesis.**

Mutant name	Amino acid residues			
	–1	31	156	166
WT	Y	I	E	G
L31	Y	L	E	G
K166	Y	L	E	K
R166	Y	L	E	R
QK	Y	L	Q	K
QR	Y	L	Q	R
D(–1)	D	L	E	G
E(–1)	E	L	E	G
DK	D	L	E	K
DR	D	L	E	R
EK	E	L	E	K
ER	E	L	E	R
DQK	D	L	Q	K
DQR	D	L	Q	R
EQK	E	L	Q	K
EQR	E	L	Q	R

WT denotes wild-type SBT. L31 was reported previously (*18*).

RESULTS

Construction of Mutant SBTs with Altered Substrate Specificity—To detect even a slight change in proteolytic activity, plasmids carrying mutant SBTs with altered substrate specificity were constructed based on the L31 mutant. L31, which has Leu in place of Ile at position 31, has increased specific activity due to its larger k_{cat} value than wild-type SBT and no apparent changes in substrate specificity (*18*). Since neither L31 nor wild-type SBT hardly cleaves peptide bonds on the C-terminal side of acid residues, Asp and Glu, we attempted to obtain mutants preferring acid residues as P1 substrates. To endow the P1 substrate-binding pocket with affinity for acid residues by changing the electrostatic environment, we replaced the Gly166 residue at the bottom of the pocket with Lys or Arg, and Glu156 with Gln based upon the reports on subtilisin BPN′ of Wells *et al.* (*16*) using site-directed mutagenesis. The resultant mutants with G166K, G166R, E156Q/G166K, or E156Q/G166R replacements were designated as K166, R166, QK, and QR, respectively, as summarized in Table I.

Because *B. subtilis* cells can be transformed only at much lower efficiency than *E. coli*, the latter is much more convenient for construction, expression and evaluation of many mutant genes. Therefore, we used the *E. coli* expression system described previously (*8–11*). As the mutant genes were inserted into the pIN-III-ompA vector, the mutant SBTs would be synthesized as prepro-form in *E. coli* cells with the signal peptide for OmpA attached to the N-terminus. Then, the prepro-SBTs would be secreted into the peri-

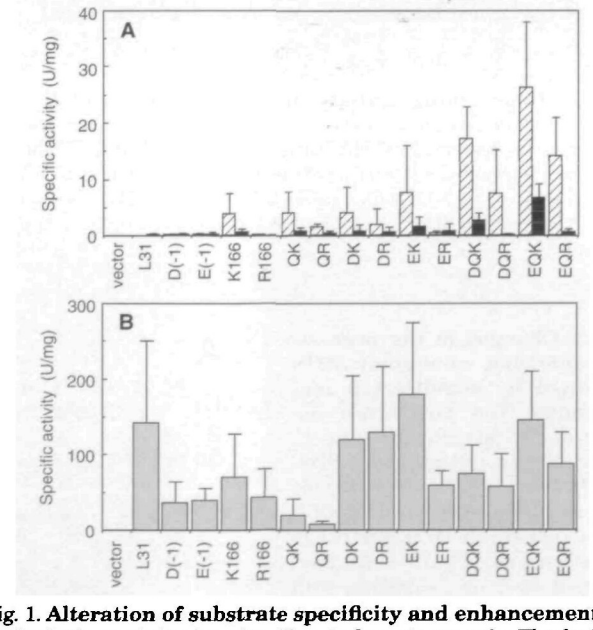

Fig. 1. **Alteration of substrate specificity and enhancement of hydrolytic activity by site-directed mutagenesis.** The hydrolytic activities for synthetic substrates, AAPE [hatched bar in (A)], AAPD [filled bar in (A)] and AAPF (B), of the mutants obtained by site-directed mutagenesis were measured as described in "MATERIALS AND METHODS." The periplasmic fractions were used as enzyme sources, and the activity is shown as units/mg protein. Each bar represents the mean ± SD ($n \geq 4$). The "vector" means the plasmid pIN-III-ompA.

plasmic space to become pro-SBTs, where they would cleave their own pro-sequence yielding the mature form (*12*). Therefore, the periplasmic fraction was prepared from *E. coli* expressing K166, R166, QK, and QR mutants and used as crude enzyme in hydrolytic assay for synthetic substrates. As shown in Fig. 1A, all mutants except R166 hydrolyzed both AAPE and AAPD, although the original L31 scarcely cleaved either AAPE or AAPD, suggesting that alteration of the specificity profile was successful. On the other hand, AAPF-hydrolytic activity was reduced in the four mutants as compared with L31 (Fig. 1B). A similar tendency was also observed in halo-forming activity on skim milk-containing plates, and no halo was observed for QK or QR after incubation for 20 h at 23°C, although they formed visible halos after 48 h at 23°C (Fig. 2).

Decreased Processing Efficiency of K166, R166, QK, and QR—Pro-enzymes of L31 and wild-type SBT must cleave the peptide bond on the C-terminal side of their own Tyr(–1) residue to become the mature form. To investigate whether the diminished AAPF-hydrolytic and halo-forming activity of the mutant SBTs with altered P1 specificity was associated with a decrease in the processing efficiency, we investigated the amounts of mature mutants in the periplasmic fractions by SDS-PAGE and immunoblotting. As shown in Fig. 3A, only small amounts of mature forms of K166, R166, QK, and QR mutants were detected, in contrast to L31 and wild-type SBT. These observations suggested that alteration of the substrate specificity profile was responsible for the decrease in processing efficiency, AAPF-hydrolysis and halo-forming activities.

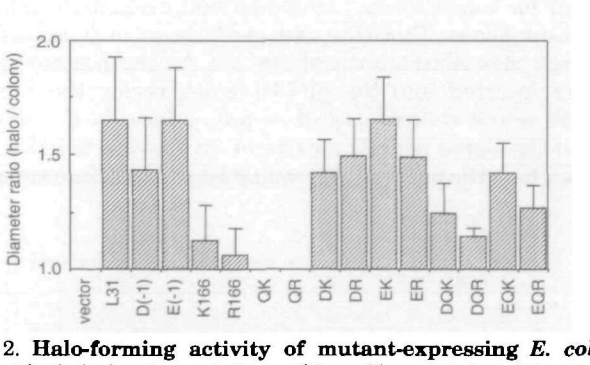

Fig. 2. **Halo-forming activity of mutant-expressing *E. coli* cells.** The halo-forming activity on skim milk-containing plates was measured as described in "MATERIALS AND METHODS." The activity was expressed as the ratio of the diameter of the halo to colony. Note that a value of 1.0 indicates no halo formation. Each bar represents the mean ± SD ($n \geq 4$). The "vector" means the plasmid pIN-III-ompA.

Introduction of Y(–1)D/E Substitution—As the mutant SBTs hydrolyzed AAPD and AAPE (Fig. 1), we attempted to improve the depressed processing efficiency by replacing the C-terminal residue in the pro-region, *i.e.*, Tyr(–1), with an acid residue, Asp or Glu, yielding DK, EK, DR, ER, DQK, EQK, DQR, and EQR mutants (Table I). As shown in Fig. 2, the introduction of Y(–1)D/E markedly enhanced halo-forming activity as compared with the corresponding parent mutants. The enhancing effect of Y(–1)E replacement seemed to be larger than that of Y(–1)D, and mutants carrying G166K substitution exhibited higher activity than those carrying G166R. Similar results were obtained by hydrolytic assay with synthetic substrates using periplasmic enzymes (Fig. 1). The profile of AAPF-hydrolysis among the mutants was again roughly consistent with the halo-forming activity (Figs. 1B and 2). In contrast, the profile of AAPD- and AAPE-hydrolytic activity was somewhat different, and the mutants derived from QK and QR showed greater activity than those derived from K166 and R166, implying that the E156Q replacement augmented the preference for these substrates (Fig. 1A).

To examine the change in processing efficiency, we again investigated the amounts of the mature enzymes in the periplasmic fractions by SDS-PAGE and immunoblotting. The detected bands of mature mutant SBTs shown in Fig. 3 were consistent with their activities (Figs. 1 and 2), suggesting that the introduction of Y(–1)D/E substitution augmented cleavage efficiency of their pro-sequence, resulting in the enhanced halo-formation and synthetic substrate-hydrolysis (Figs. 1–3).

Measurement of Kinetic Parameters for EQK and EQR—Although the periplasmic mutant enzymes showed a clear alteration of P1 substrate preference for acid residues, the specificity change can be expressed more accurately by measuring kinetic parameters. As EQK and EQR mutants showed the highest apparent activity for both AAPE and AAPD among the corresponding mutant series harboring

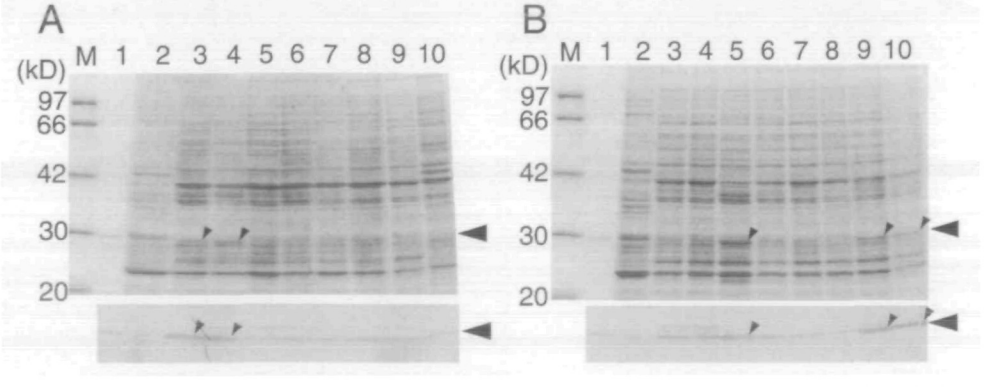

Fig. 3. **Changes in the processing efficiency of mutant SBTs obtained by site-directed mutagenesis.** The periplasmic proteins of the mutant-expressing *E. coli* cells were separated by SDS-PAGE using 12.5% polyacrylamide gels and either stained with CBB R-250 (upper panels) or electrically transferred onto PVDF membranes followed by immunostaining with anti-mature SBT antiserum (lower panels). Lane 1 in both (A) and (B), standard purified sample of mature wild-type SBT; lane 2 in both (A) and (B), periplasmic fraction prepared from cells harboring pIN-III-ompA. Lanes 3–10, periplasmic fractions were prepared from cells expressing wild-type SBT, L31, D(–1), E(–1), K166, R166, QK, and QR, respectively in (A), while DK, DR, EK, ER, DQK, DQR, EQK, and EQR, respectively in (B). The molecular weight marker is designated as M. The fractions containing large amounts of mature enzymes exhibited strong bands (small arrowheads in upper and lower panels). The large arrowheads indicate the positions of mature enzymes.

G166K and G166R replacements, respectively, we investigated kinetic parameters for these mutants using purified enzymes.

Because *B. subtilis* cells could secrete a larger amount of protein than *E. coli*, the mutant enzymes for purification were produced by *B. subtilis*. To construct plasmids expressing EQK, EQR, and L31 in *B. subtilis*, the *Nde*I- and *Bam*HI-digested insert of pIOE (*11*) harboring the wild-type SBT gene inserted in a pHY300PLK was exchanged with those of the EQK, EQR, and L31 genes, respectively. *B. subtilis* DB403, a mutant strain with low protease activity, was transformed with the constructed plasmids, and active enzymes were purified from the culture supernatant to homogeneity by cation-exchange chromatography. With the purified enzymes, k_{cat} and K_m values for the hydrolysis of AAPF, AAPE, and AAPD were determined as shown in Table II. While the specific activity for AAPF was not significantly changed, EQK and EQR exhibited markedly enhanced hydrolytic activity for AAPE and AAPD, which were little cleaved by L31. Therefore, it was confirmed that the substrate specificity profile was changed, and the P1 substrate preferences of the mutants were extended to acid residues. In addition, AAPK-hydrolytic activity of the purified EQK and EQR was 40 ± 10 and 26 ± 10 U/mg (mean ± SD, n = 3), respectively, markedly lower than that of L31 [950 ± 20 U/mg (mean ± SD, n = 3)]. This seemed to be compatible with the reports on subtilisin BPN′ by Wells *et al.* (*16*).

Determination of N-Terminal Sequences of EQK and EQR—It is necessary to investigate whether a mutant with altered specificity was really autoprocessed at the correct site. As the pro-enzymes of EQK and EQR had Glu residues at both positions –2 and –1, N-terminal sequencing of their mature forms was especially important. The N-terminal sequences of EQK and EQR that had been purified to homogeneity were determined to be the same as that of L31, Ala-Gln-Ser-Val-Pro-Tyr-, and a single peak of phenylthiohydantoin–amino acid was given at every cycle of the Edman degradation, confirming that the pro-enzymes of these mutants were cleaved on the C-terminal side of Glu(–1).

Quantitative Analysis of Periplasmic Mature Enzymes—To support the result shown in Fig. 3, we attempted to estimate the amount of mature enzymes in the periplasmic fraction of the mutants. The specific activity of the purified enzymes of L31, EQK, and EQR for AAPF-hydrolysis was measured as 4,600 ± 140, 7,100 ± 680, and 5,800 ± 1,100 U/mg (mean ± SD, n = 3–4), respectively. Assuming that the specific activity of purified QK and QR was the same as that of EQK and EQR, respectively, the amounts of the mature enzymes in the periplasmic fractions were calculated from the values described in Fig. 1B. Thus, the average amounts in the periplasmic fractions of L31, QK, QR, EQK, and EQR were estimated as 31 ± 24, 2.6 ± 3.1, 1.2 ± 0.94, 20 ± 9.4, and 15 ± 7.5 μg per 1 mg of total protein (mean ± SD, n = 4–8), respectively. Thus, the profile of these calculated amounts seemed to be roughly compatible with the detected bands in Fig. 3.

Introduction of Random Mutation in the Pro-Region of QK—As described above, the introduction of Y(–1)D/E substitution was very effective for improving the depressed processing efficiency of the mutant SBTs. However, to search randomly for other substitutions in the pro-region bringing larger improvements, we performed error-prone PCR in the pro-region as described in "MATERIALS AND METHODS." To screen a large amount of clones by halo-forming assay, QK or QR mutant was a suitable template, because the colonies of *E. coli* expressing QK or QR showed no halo formation after 20 h of incubation at 23°C (Fig. 2). Moreover, EQK exhibited the largest halos among the mutants derived from QK and QR obtained by site-directed mutagenesis (Fig. 2). Therefore, QK seemed the most appropriate for introduction of random mutations and screening.

TABLE II. **Kinetic constants of L31 and mutants with altered P1 specificity for the hydrolysis of synthetic peptide substrates.**

Substrate	Mutant	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
AAPF	L31	240 ± 25	1.5 ± 0.2	160 ± 5
	EQK	280 ± 58	1.1 ± 0.2	250 ± 11
	EQR	170 ± 30	1.0 ± 0.1	170 ± 15
AAPE	L31	N.D.	N.D.	N.D.
	EQK	32 ± 3	0.37 ± 0.04	87 ± 10
	EQR	22 ± 2	0.46 ± 0.07	48 ± 12
AAPD	L31	N.D.	N.D.	N.D.
	EQK	2.8 ± 1.2	0.44 ± 0.07	6.3 ± 1.6
	EQR	0.087 ± 0.005	0.083 ± 0.014	1.1 ± 0.1

The enzymatic reaction was carried out in 50 mM Tris-HCl (pH 8.5) and 1 mM $CaCl_2$ at 37°C using purified enzymes. The data are means ± SD (n = 4).

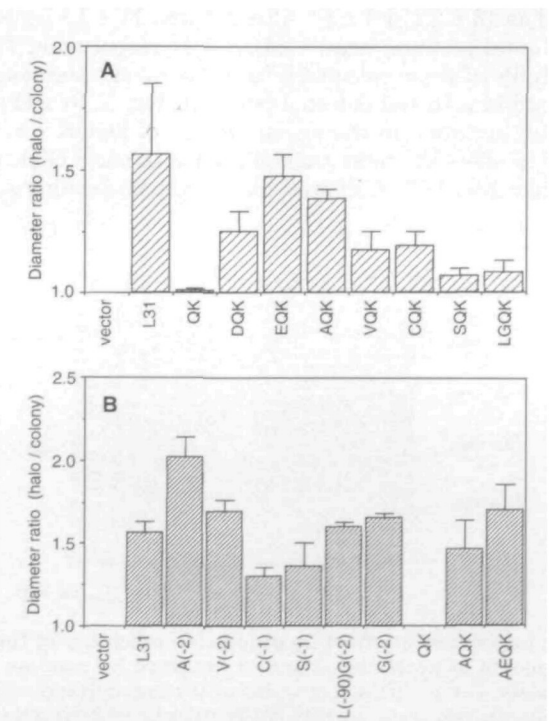

Fig. 4. **Analyses of improvement effect on halo-forming activity of the substitutions at positions –2 and –1, obtained by site-directed and random mutageneses.** The halo-forming assay was performed as described in "MATERIALS AND METHODS." (A) Comparison of the mutant-expressing *E. coli* cells obtained by random mutagenesis with the cells expressing Y(–1)D/E-substituted QK. (B) Substitutions were introduced at positions –2 and –1 in the pro-region of L31, and the specificity of the improvement effect on halo-forming activity to the mutant QK was examined. The cumulative nature of the effects of E(–2)A and Y(–1)E for QK was also examined. The "vector" means the plasmid pIN-III-ompA.

Eleven colonies showing obvious caseinolytic activity after 20 h at 23°C were isolated by screening of approximately 16,000 colonies. Plasmid DNA was prepared from each clone and used for re-transformation of *E. coli* to confirm that the enhanced halo-forming activity was due to the plasmid itself, resulting in nine clones. By sequencing the region of the nine mutants amplified by error-prone PCR, three clones each of AQK and VQK and one clone each of CQK, SQK, and LGQK were identified (Fig. 4A). All mutations originated from a 1 nt change, and most replacements in amino acid residues were localized at position –2 or –1: AQK and VQK had E(–2)A and E(–2)V replacements, respectively, CQK and SQK had Y(–1)C and Y(–1)S, respectively, and LGQK had mutations at two positions, E(–2)G in the pro-region and F(–90)L in the signal peptide. These mutants showed halo-to-colony diameter ratios of about 1.1 to 1.4 in contrast to QK (Fig. 4A). Moreover, SDS-PAGE and immunoblotting detected increased amounts of the mature enzymes in the periplasmic fraction prepared from cells expressing the mutants, especially AQK, indicating that the processing efficiency was enhanced in these pro-region mutants (Figs. 4A and 5). By assuming that the specific activity of purified QK and AQK for AAPF-hydrolysis was the same as that of purified EQK, the effect of the E(–2)A substitution could be compared with that of Y(–1)E by quantitative estimation of the amounts of mature enzymes in the periplasmic fractions. The average amounts of the mature enzymes of L31, QK, EQK, and AQK were estimated as 16 ± 2.1, 4.3 ± 1.1, 12 ± 2.7, and 11 ± 1.1 μg per 1 mg of total protein (mean ± SD, n = 4), respectively. Thus, the profile of these calculated values seemed to be roughly compatible with the detected bands in Fig. 5. In addition, F(–90)L mutation in the signal peptide of LGQK was unlikely to affect the processing efficiency, because GQK constructed from LGQK showed the same halo-forming activity as LGQK.

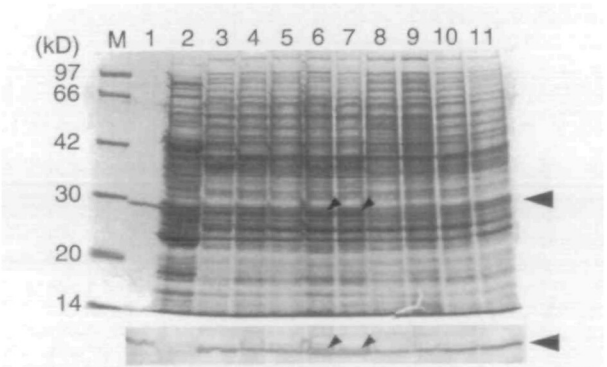

Fig. 5. **Improvement effect on processing efficiency of the replacements at positions –2 and –1 obtained by random mutagenesis.** The periplasmic proteins of the mutant-expressing *E. coli* cells were separated by SDS-PAGE using 12.5% polyacrylamide gel and either stained with CBB R-250 (upper panel) or blotted onto PVDF membranes and probed with anti-mature SBT antiserum (lower panel). Lane 1, standard purified sample of mature wild-type SBT; lane 2, periplasmic fraction prepared from cells harboring pIN-III-ompA; lanes 3–11, periplasmic fractions prepared from cells expressing L31, QK, DQK, EQK, AQK, VQK, CQK, SQK, and LGQK, respectively. M, molecular weight marker. The fractions containing large amounts of mature enzymes showed strong bands in both the CBB-stained gel and the immunostained membrane (small arrowheads in upper and lower panels, respectively). The large arrowheads indicate the positions of mature enzymes.

Replacement of Glu(–2) Affects L31 Itself—To determine whether the enhancement effect of the substitutions at position –2 and –1 was specific to QK, the fragment containing both pre- and pro-regions of L31, the basal mutant of all the mutant SBTs in this study, was exchanged with those of AQK, VQK, CQK, SQK, and LGQK as described in "MATERIALS AND METHODS," yielding A(–2), V(–2), C(–1), S(–1), and L(–90)G(–2) mutants, respectively. To test the effects of F(–90)L substitution, the fragment including only the pro-region of L31 was also exchanged with that of LGQK, resulting in G(–2). As shown in Fig. 4B, A(–2), V(–2), L(–90)G(–2), and G(–2) mutants formed slightly or much larger halos than L31, whereas C(–1) and S(–1) showed smaller halos. Therefore, the mutations at position –1 seemed to enhance the processing efficiency of QK specifically, while mutations at position –2 seemed to augment that of L31 itself. Moreover, when the Y(–1)E mutation was introduced into AQK by site-directed mutagenesis, a larger halo was formed by the resultant clone AEQK as shown in Fig. 4B, suggesting that the effect of E(–2)A replacement was independent of and cumulative with that of Y(–1)E.

DISCUSSION

There have been many reports of success in creating new enzymes with altered substrate specificity in the field of protein engineering based on well-studied enzymes including members of the subtilisin family (*23–25*). Wells *et al.* demonstrated that G166K-, G166R-, or E156Q/G166K-substituted mutants of subtilisin BPN′ showed increased preference for the Glu residue as the P1 substrate (*16*). Our observations of the AAPD- and AAPE-hydrolytic activities of K166, R166, QK, and QR mutant SBTs, which were measured with periplasmic enzymes prepared from *E. coli* cells, seemed to be compatible with those of Wells *et al.* However, the cells expressing our mutants showed marked decreases in halo-forming and AAPF-hydrolytic activity, and moreover, only small amounts of mature enzymes were detected in the periplasmic fractions, suggesting that the processing efficiency of our mutants was depressed. As SBT is an autoprocessing enzyme and autocatalytically cleaves its own pro-sequence at the peptide bond on the C-terminal side of Tyr(–1) to become the mature form, the decrease in the processing efficiency seemed to be due to the altered P1 substrate specificity (*26*). Moreover, the X-ray crystal structure of the complex of autoprocessed Ser221Cys-SBT with its pro-region indicated that the C-terminus of the pro-region binds to the active site in a product-like manner, as the Tyr(–1) residue was held in the P1 substrate-binding pocket (*27*). Therefore, we first introduced Y(–1)D/E replacement by site-directed mutagenesis and succeeded in improving the decreased processing efficiency, which was demonstrated by the enhanced activity of halo formation and synthetic substrate hydrolysis by periplasmic enzymes and increased amounts of mature enzymes detected in the periplasm (Figs. 1–3). In addition, we confirmed the hydrolytic activity of the purified mutant enzymes for not only AAPE but also AAPD, although the latter substrate was less preferred (Table II). Moreover, it should be emphasized that the N-terminal sequences of the purified enzymes from EQK- and EQR-expressing *B. subtilis* were uniform and the same as that of L31, Ala-Gln-Ser-Val-Pro-Tyr-, con-

firming that the pro-enzymes of these mutants were both cleaved after Glu(–1). These results not only confirmed that P1 substrate specificity was extended to acid residues but also supported the appropriateness of the introduction of Y(–1)D/E substitutions. In addition, it is noteworthy that the P1 substrate specificity for Phe of the purified EQK and EQR are not changed very much (Table II), although the processing efficiency on the C-terminal side of Tyr(–1) is decreased. Because the P1 pockets of QK and QR are deduced to be shallow due to E156Q and G166K/R replacements, the hydroxyl group in the side chain of Tyr(–1) might cause steric hindrance.

Next, we performed error-prone PCR in the pro-region of QK and isolated five independent mutants with improved processing efficiency (Fig. 5). All the mutants possessed replacements at position –2 or –1, *i.e.*, E(–2)A/V/G or Y(–1)C/S. The E(–2)A replacement exhibited the most significant enhancement effect among the random mutations, although the extent of this effect was less than that of Y(–1)E. Exchange of the pro-region of L31 with that of the E(–2)X and Y(–1)X series of the mutants caused formation of larger and smaller halos than L31, respectively, indicating that the improvement effect of the Y(–1)C/S mutation was specific to QK, and that of E(–2)A/V/G was, rather, general to the L31-derived mutants. Although the effect of Y(–1)C/S was specific to QK, it could not be explained by an electrostatic interaction between the residue at position –1 and the P1 pocket. As the P1 pocket of QK was deduced to be shallow due to E156Q and G166K replacements, the rather weak effect of Y(–1)C/S may have been due to alleviation of steric hindrance, because the side chains of Cys and Ser are much smaller than Tyr. This was supported by our previous observation that the Y(–1)A substitution improved the depressed processing caused by replacement of Gly127, a residue at the entrance of the P1 pocket, with Ala or Val, resulting in restriction of the P1 preference for Ala, a residue with a small side chain (*9*).

It is essential to investigate whether a mutant with altered specificity is autoprocessed at the correct site. In immunoblotting analyses, extra bands were detected at larger molecular size than the mature mutant enzymes indicated by arrowheads (Figs. 3 and 5). There are two possible explanations for their occurrence: generation of other mature forms by irregular autoprocessing, and degradation in the periplasm of mutant pro-enzymes by active mature ones generated by correct autoprocessing. The latter could occur when the binding of pro- and mature-regions in a mutant pro-SBT was rather unstable as compared with wild-type SBT and L31. Therefore, to determine whether the mutant pro-SBTs were correctly autoprocessed, the N-terminal sequences of the purified active enzymes were investigated. Although the sequences of DK, DR, EK, ER, DQK, and DQR were not examined, the N-terminal sequences of active mature enzymes of EQK and EQR, which have the highest hydrolytic activity for AAPE and AAPD among the site-directed mutants, were uniform and the same as that of L31. Therefore, the pro-regions of at least EQK and EQR were definitely processed on the C-terminal side of Glu(–1), indicating that the correctly-autoprocessed form was probably responsible for the hydrolysis activity (Fig. 1) and was detected as the mature form (Fig. 3). As for the random mutants, AQK showed the highest activity, though the activity was little less than that of EQK (Fig. 4A). However, the E(–2)A replacement enhanced that of L31 itself (Fig. 4B). Therefore, the mechanism of the enhancement effect of E(–2)A substitution is now being investigated with A(–2), V(–2), and G(–2) mutants shown in Fig. 4B. The N-terminal sequences of the purified enzymes prepared from the A(–2)-, V(–2)-, and G(–2)-expressing *B. subtilis* were also determined. They were uniform and the same as that of L31, Ala-Gln-Ser-Val-Pro-Tyr-, indicating that the pro-regions of these mutants were cleaved correctly at position –1.

The improvement effect of the mutations at positions –2 and –1 seemed to be independent, because AEQK harboring both E(–2)A and Y(–1)E substitutions caused formation of larger halos than AQK (Fig. 4B). Interestingly, the amino acid residues at position –2 are also Ala in subtilisins BPN′ and Carlsberg, which are members of the subtilisin family phylogenetically close to SBT (Fig. 6) (*28*). As the processing efficiency and productivity of mature active enzymes are higher than those of SBT (Takagi, H., unpublished observation), the Ala(–2) residue may be involved in efficient processing. The X-ray crystal structure of the complex of SBT with its pro-region shows that the Glu(–2) residue is adjacent to the His64 residue, a member of the catalytic triad of SBT (*27*). It was reported that H64A-substituted subtilisin BPN′ could partially recover the function of the lost catalytic His64 from a His residue at the P2 site in a substrate by the mechanism of substrate-assisted catalysis (*29*). Moreover, when the Glu residue at position –2, the site corresponding to the P2 site in a substrate, was replaced with His, the lost autoprocessing activity of H64A-substituted pro-SBT was restored by a similar mechanism to the substrate-assisted catalysis (*30*). In these cases, His residues at positions P2 and –2 mimicked the lost His64 and reconstituted a new catalytic triad with Asp32 and Ser221. Therefore, E(–2)A replacement in this study might cause the conformational change of the adjacent His64 residue, resulting in enhancement of autoprocessing. However, the detailed mechanism by which the mutations at the position –2 function remains to be investigated.

The pro-region of SBT is essential for appropriate folding of the mature region (*15*), and complementary fitting between the C-terminus of the pro-region and the substrate-binding site of the mature region seems to be an important factor for efficient autoprocessing (*9*). We demonstrated that engineering in the pro-region was a useful method for improving the appropriate processing of mutant SBTs with altered specificity. Although we employed

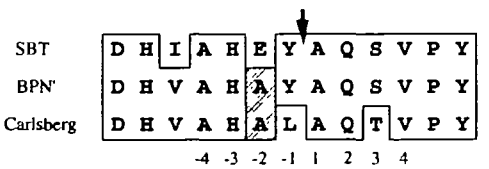

Fig. 6. Conservation of the Ala(–2) residue in the pro-region of subtilisins BPN′ and Carlsberg. The sequence around the auto-cleavage site of pro-SBT was aligned with those of subtilisins BPN′ and Carlsberg, two members of the subtilisin family phylogenetically close to SBT. The identical residues are shown in boxes and the conserved Ala(–2) residue is shown in a shaded box. The arrow marks the scissile bond. The data are from GenBank/EMBL Data Bank. The accession numbers are K01988 (SBT), X00165 (BPN′), and X03341 (Carlsberg).

mutants with increased P1 preference for acid residues, a similar approach is applicable to other mutants of autoprocessing proteases with differentially altered specificity, including α-lytic protease (*6, 31*) and aqualysin I (*32, 33*). One such example is a variant of subtilisin BPN′ engineered by Ballinger *et al.*, whose substrate specificity was changed so that it cleaved a furin-like tribasic substrate (*25*). They found that the decreased amount of the mature variant was increased by changing the C-terminal sequence of the wild-type pro-region into a substrate-like sequence. In addition, we investigated in this study the enhancement effect of engineering in the pro-region of mutant SBTs on the autoprocessing efficiency using the *E. coli* expression system for efficient screening. However, it may be also important to evaluate the effect of the substitutions in the *B. subtilis* expression system and compare the characteristics of the two systems.

It is potentially very useful to develop a number of proteases as molecular tools with various types of substrate specificity for both basic and applied sciences. The recent progress in protein engineering techniques has allowed generation of mutants with various beneficial properties including altered substrate specificity. In this context, our study suggests the usefulness of "pro-sequence engineering" for developing variants of autoprocessing proteases.

REFERENCES

1. Leis, J.P. and Cameron, C.E. (1994) Engineering proteases with altered specificity. *Curr. Opin. Biotechnol.* **5**, 403–408
2. Perona, J.J. and Craik, C.S. (1995) Structural basis of substrate specificity in the serine proteases. *Protein Sci.* **4**, 337–360
3. Perona, J.J. and Craik, C.S. (1997) Evolutionary divergence of substrate specificity within the chymotrypsin-like serine protease fold. *J. Biol. Chem.* **272**, 29987–29990
4. Kurth, T., Grahn, S., Thormann, M., Ullmann, D., Hofmann, H.J., Jakubke, H.D., and Hedstrom, L. (1998) Engineering the S1′ subsite of trypsin: Design of a protease which cleaves between dibasic residues. *Biochemistry* **37**, 11434–11440
5. Ikemuram, H. and Inouye, M. (1988) In vitro processing of prosubtilisin produced in *Escherichia coli*. *J. Biol. Chem.* **263**, 12959–12963
6. Silen, J.L. and Agard, D.A. (1989) The alpha-lytic protease proregion does not require a physical linkage to activate the protease domain in vivo. *Nature* **341**, 462–464
7. Zhu, X.L., Ohta, Y., Jordan, F., and Inouye, M. (1989) Prosequence of subtilisin can guide the refolding of denatured subtilisin in an intermolecular process. *Nature* **339**, 483–484
8. Takagi, H., Maeda, T., Ohtsu, I., Tsai, Y.C., and Nakamori, S. (1996) Restriction of substrate specificity of subtilisin E by introduction of a side chain into a conserved glycine residue. *FEBS Lett.* **395**, 127–132
9. Takagi, H., Ohtsu, I., and Nakamori, S. (1997) Construction of novel subtilisin E with high specificity, activity and productivity through multiple amino acid substitutions. *Protein Eng.* **10**, 985–989
10. Takagi, H., Yamamoto, M., Ohtsu, I., and Nakamori, S. (1998) Random mutagenesis into the conserved Gly154 of subtilisin E: isolation and characterization of the revertant enzymes. *Protein Eng.* **11**, 1205–1210
11. Takagi, H., Hirai, K., Maeda, Y., Matsuzawa, H., and Nakamori, S. (2000) Engineering subtilisin E for enhanced stability and activity in polar organic solvents. *J. Biochem.* **127**, 617–625
12. Ikemura, H., Takagi, H., and Inouye, M. (1987) Requirement of pro-sequence for the production of active subtilisin E in *Escherichia coli*. *J. Biol. Chem.* **262**, 7859–7864
13. Shinde, U. and Inouye, M. (1994) The structural and functional organization of intramolecular chaperones: The N-terminal propeptides which mediate protein folding. *J. Biochem.* **115**, 629–636
14. Shinde, U., Fu, X., and Inouye, M. (1999) A pathway for conformational diversity in proteins mediated by intramolecular chaperones. *J. Biol. Chem.* **274**, 15615–15621
15. Fu, X., Inouye, M., and Shinde, U. (2000) Folding pathway mediated by an intramolecular chaperone. *J. Biol. Chem.* **275**, 16871–16878
16. Wells, J.A., Powers, D.B., Bott, R.R., Graycar, T.P., and Estell, D.A. (1987) Designing substrate specificity by protein engineering of electrostatic interactions. *Proc. Natl. Acad. Sci. USA* **84**, 1219–1223
17. Siezen, R.J. and Leunissen, J.A.M. (1997) Subtilases: The superfamily of subtilisin-like serine proteases. *Protein Sci.* **6**, 501–523
18. Takagi, H., Morinaga, Y., Ikemura, H., and Inouye, M. (1988) Mutant subtilisin E with enhanced protease activity obtained by site-directed mutagenesis. *J. Biol. Chem.* **263**, 19592–19596
19. Koshland, D. and Botstein, D. (1980) Secretion of β-lactamase requires the carboxy end of the protein. *Cell* **20**, 749–760
20. Ohse, M., Takahashi, K., Kadowaki, Y., and Kusaoke, H. (1995) Effects of plasmid DNA sizes and several other factors on transformation of *Bacillus subtilis* ISW1214 with plasmid DNA by electroporation. *Biosci. Biotechnol. Biochem.* **59**, 1433–1437
21. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685
22. Towbin, H., Staehelin, T., and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354
23. Rheinnecker, M., Baker, G., Eder, J., and Fersht, A.R. (1993) Engineering a novel specificity in subtilisin BPN′. *Biochemistry* **32**, 1199–1203
24. Bech, L.M., Sorensen, S.B., and Breddam, K. (1993) Significance of hydrophobic S_4-P_4 interactions in subtilisin 309 from *Bacillus lentus*. *Biochemistry* **32**, 2845–2852
25. Ballinger, M.D., Tom, J., and Wells, J.A. (1996) Furilisin: A variant of subtilisin BPN′ engineered for cleaving tribasic substrates. *Biochemistry* **35**, 13579–13585
26. Shinde, U. and Inouye, M. (1995) Folding pathway mediated by an intramolecular chaperone: characterization of the structural changes in pro-subtilisin E coincident with autoprocessing. *J. Mol. Biol.* **252**, 25–30
27. Jain, S.C., Shinde, U., Li, Y., Inouye, M., and Berman, H.M. (1998) The crystal structure of an autoprocessed Ser221Cys-subtilisin E-propeptide complex at 2.0 Å resolution. *J. Mol. Biol.* **284**, 137–144
28. Siezen, R.J., de Vos, W.M., Leunissen, J.A.M., and Dijkstra, B.W. (1991) Homology modelling and protein engineering strategy of subtilases, the family of subtilisin-like serine proteinases. *Protein Eng.* **4**, 719–737
29. Carter, P. and Wells, J.A. (1987) Engineering enzyme specificity by "substrate-assisted catalysis." *Science* **237**, 394–399
30. Shinde, U. and Inouye, M. (1995) Folding mediated by an intramolecular chaperone: autoprocessing pathway of the precursor resolved via a substrate assisted catalysis mechanism. *J. Mol. Biol.* **247**, 390–395
31. Silen, J.L., Frank, D., Fujishige, A., Bone, R., and Agard, D.A. (1989) Analysis of prepro-α-lytic protease expression in *Escherichia coli* reveals that the pro region is required for activity. *J. Bacteriol.* **171**, 1320–1325
32. Lee, Y.C., Miyata, Y., Terada, I., Ohta, T., and Matsuzawa, H. (1991) Involvement of NH_2-terminal pro-sequence in the production of active aqualysin I (a thermophilic serine protease) in *Escherichia coli*. *Agric. Biol. Chem.* **55**, 3027–3032
33. Terada, I., Kwon, S.T., Miyata, Y., Matsuzawa, H., and Ohta, T. (1990) Unique precursor structure of an extracellular protease, aqualysin I, with NH_2- and COOH-terminal pro-sequences and its processing in *Escherichia coli*. *J. Biol. Chem.* **265**, 6576–6581