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Engineering Subtilisin E for Enhanced Stability and Activity in Polar Organic Solvents[1]

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We examined the effect of a novel disulfide bond engineered in subtilisin E from *Bacillus subtilis* based on the structure of a thermophilic subtilisin-type serine protease aqualysin I. Four sites (Ser163/Ser194, Lys170/Ser194, Lys170/Glu195, and Pro172/Glu195) in subtilisin E were chosen as candidates for Cys substitutions by site-directed mutagenesis. The Cys170/Cys195 mutant subtilisin formed a disulfide bond in *B. subtilis*, and showed a 5–10-fold increase in specific activity for an authentic peptide substrate for subtilisin, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide, compared with the single-Cys mutants. However, the disulfide mutant had a 50% decrease in catalytic efficiency due to a smaller k_{cat} and was thermolabile relative to the wild-type enzyme, whereas it was greatly stabilized relative to its reduced form. These results suggest that an electrostatic interaction between Lys170 and Glu195 is important for catalysis and stability in subtilisin E. Interestingly, the disulfide mutant was found to be more stable in polar organic solvents, such as dimethylformamide and ethanol, than the wild-type enzyme, even under reducing conditions; this is probably due to the substitution of uncharged Cys by charged surface residues (Lys170 and Glu195). Further, the amino-terminal engineered disulfide bond (Gly61Cys/Ser98Cys) and the mutation Ile31Leu were introduced to enhance the stability and catalytic activity. A prominent 3–4-fold increase in the catalytic efficiency occurred in the quintet mutant enzyme over the range of dimethylformamide concentration (up to 40%).

Key words: aqualysin I, disulfide bond, site-directed mutagenesis, stability in organic solvents, subtilisin E.

Subtilisin, a cysteine-free alkaline serine protease produced by various *Bacillus* species, has been extensively investigated as a promising target for protein engineering (*1–5*). For instance, many attempts have been made to enhance its thermostability by the introduction of unnatural intramolecular disulfide bond(s). There are conflicting reports regarding the thermostability of subtilisin BPN′ in which a designed disulfide bond has been introduced with the aid of computer modeling between positions 22 and 87 (*6*, *7*). Furthermore, based upon molecular modeling, five other disulfide variants of subtilisin BPN′ have been constructed (Cys26/Cys232, Cys29/Cys119, Cys36/Cys210, Cys41/Cys80, and Cys148/Cys243) (*8*, *9*). These disulfides were chosen on the basis of structural homology with proteinase K, a fungal protease that contains two disulfide bonds. In some cases, the disulfide bond–containing protein was stabilized relative to its reduced form. However, none of the mutants were dramatically stabilized by introducing a *de novo* designed disulfide bridge, indicating that finding the positions in folded globular proteins capable of accomodating an unstrained disulfide bond is not a clear-cut task.

Using an *Escherichia coli* expression system, we engineered *Bacillus subtilis* I168 subtilisin E (*10–14*). In our previous work (*11*), we investigated the thermostability of subtilisin E after the introduction of a disulfide that was engineered based on structural similarity to a thermophilic subtilisin-type serine protease, aqualysin I of *Thermus aquaticus* YT-1 (*15*). Aqualysin I contains two disulfide bonds, Cys67/Cys99 and Cys163/Cys194, that seem to be responsible for the thermostability (*16*, *17*). Gly61 and Ser98 in subtilisin E were each replaced with Cys, corresponding to the Cys residues at positions 67 and 99 of aqualysin I, by site-directed mutagenesis. We found that the Cys61/Cys98 mutant subtilisin appears to form a disulfide bond spontaneously in the *E. coli* expression system and that the N-terminal disulfide bond enhances the thermostability of subtilisin E without any change in its catalyic efficiency (*11*). Recently, other similar studies involving other alkaline proteases from *Aspergillus oryzae* (*18*) and

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Abbreviations: AAPF, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide; β-ME, β-mercaptoethanol; DTT, dithiothreitol; IPTG, isopropyl-β-D-thiogalactopyranoside; PCR, polymerase chain reaction; DSC, differential scanning calorimetry; CD, circular dichroism; DMF, dimethylformamide; SDS-PAGE, SDS–polyacrylamide gel electrophoresis.

Pseudomonas sp. (*19*) have been successful on the basis of structural comparison with aqualysin I.

In the present study, we focused on the C-terminal disulfide bond in aqualysin I, corresponding to the Cys163/Cys194, and constructed mutant subtilisins E containing an engineered disulfide bond. We examined the catalytic properties and stabilities of the mutant subtilisins under various conditions including high temperature and the presence of organic solvents. It was found that the Cys170/Cys195 mutant enzyme shows improved stability in polar organic solvents, whereas the thermal stability is less than that of the wild-type enzyme. We also attempted to enhance the catalytic activity of the mutant enzyme in polar organic solvents.

In this article, a mutant subtilisin designated as K170C indicates the enzyme in which Lys170 is replaced by Cys. Mutant enzymes with multiple mutations are indicated with the mutation sites separated by a slash, *i.e.* K170C/E195C.

MATERIALS AND METHODS

Materials—An *E. coli* strain JA221 (*hsdM*$^+$ *trpE5 leuB6 lacY recA1/F lacI*^{q} *lac*$^+$ *pro*$^+$) (*20*) 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 wild-type and mutant subtilisins E. The isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible pIN-III-ompA vector (*21*) and pHY300PLK (Takara Shuzo, Kyoto) were used for the expression and secretion of subtilisins E in *E. coli* and *B. subtilis*, respectively. An *E. coli* C600 (*recA*$^+$ *F*$^-$ *thi-1 thr-1 leuB6 lacY1 tonA21 supE44* λ$^-$) was used for construction of expression plasmids for *B. subtilis*. All the enzymes for the DNA manipulations were ob-tained from Takara Shuzo. Synthetic peptide substrates were purchased from Bachem Feinchemikalien (Bubendorf, Switzerland) or Sigma (St. Louis, USA).

Site-Directed Mutagenesis—The replacement of Ser163, Lys170, Pro172, Ser194, and Glu195 by Cys was performed by polymerase chain reaction (PCR) with oligonucleotide primers, 5′-GAAGCACAT°GCACAGTC-3′ (primer 163+), 5′-GACTGTGCA°TGTGCTTC-3′ (primer 163−), 5′-TACCCTGCAT°G°C°TATCCTTCTACT-3′ (primer 170+), 5′-AGTAGAAGGATAG°C°A°TGCAGGGTA-3′ (primer 170−), 5′-CTGCAAAATATT°G°TTCTACTATTGC-3′ (primer 172+), 5′-GCAATAGTAGAAC°A°ATATTTTGCAG-3′ (primer 172−), 5′-GCAGGTTG°TGAGCTTGA-3′ (primer 194+), 5′-TCAAGCTCAC°AACCTGC-3′ (primer 194−), 5′-GCAGGTTCTT°G°T°CTTGATGTGAT-3′ (primer 195+), and 5′-ATCACATCAAGA°C°A°AGAACCTGC-3′ (primer 195−), respectively, using a Gene Amp PCR system 2400 (Perkin-Elmer Applied Biosystems, Foster City, USA). The asterisks show the locations of mismatches. A plasmid pHI212 (*10*), containing the wild-type subtilisin E gene inserted into a pIN-III-ompA, was used as a template DNA for site-directed mutagenesis. In addition, primers P100 (5′-ACAGGAAGCGGCCAATAT-3′) and P240 (5′-GTCGGGTGCTTAGAAAGA-3′) were synthesized to complement regions 57 bp upstream of the *Bst*XI restriction site and 54 bp downstream of the *Nco*I restriction site in pHI212, respectively. The expected band of the PCR product was digested with *Bst*XI and *Nco*I to recover the 316 bp fragment and ligated to an 8.3 kb fragment of plasmid pHI212 digested with *Bst*XI and *Nco*I. The mutations were confirmed with a Model 377 DNA sequencer (Perkin-Elmer Applied Biosystems) using dideoxy chain termination sequencing. The resulting plasmid was then used as a template to construct the double Cys mutant subtilisins (S163C/S194C, K170C/S194C, K170C/E195C, and P172C/E195C).

Construction of Expression Plasmids for B. subtilis—To isolate the wild-type subtilisin E gene containing the putative promoter and terminator regions by PCR, genomic DNA was prepared from *B. subtilis* I168, and the sense and the antisense primers were designed based on the nucleotide sequence outside the gene, as determined by Stahl and Ferrari (*22*). The sense primer was 5′-CGGAATTCT-ACCTAAATAGAGAT-3′ (the underlined sequence shows the position of an *Eco*RI site), and the antisense primer was 5′-GCCCTAGGGCTTGTGAAGATTTT-3′ (the underlined sequence shows the position of a *Bam*HI site). The unique amplified band of 1,437 bp was digested with *Eco*RI and *Bam*HI and then ligated to the *Eco*RI and *Bam*HI site of pHY300PLK. The nucleotide sequence was confirmed by DNA sequencing. The resulting plasmid was designated pIOE.

Four plasmids (pIO-NSS, pIO-CSS, pIO-DSS, and pIO-LDSS) carrying the disulfide mutant subtilisin E genes (G61C/S98C, K170C/E195C, G61C/S98C/K170C/E195C, and I31L/G61C/S98C/K170C/E195C), respectively, were constructed in *E. coli* C600 as described in the legend to Fig. 2. These plasmids were then introduced into *B. subtilis* DB403 by electroporation (23).

Expression and Purification of Wild-Type and Mutant Subtilisins E—Wild-type and mutant subtilisin E genes were expressed in the *E. coli* strain JA221. The periplasmic fraction was prepared by the method of Koshland and Botstein (*24*). To characterize the mutant subtilisins E, a recombinant *B. subtilis* strain was grown at 37°C for 24 h in LB medium containing tetracycline (20 μg/ml). An ammonium sulfate precipitate (70% saturation) of the culture fluid was first applied to a cation-ion exchange CM-Sepharose Fast Flow column (Pharmacia Biotech, Uppsala, Sweden), 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 by a 0–160 mM NaCl gradient was collected. The eluted active fraction showed a single protein band of subtilisin E upon SDS-PAGE. The enzyme concentration was determined spectrophotometrically by the absorbance at 280 nm using Subtilisin BPN′ (Sigma) as a standard.

Assay of Subtilisin Activity—For the synthetic peptide substrate, assays were performed as described previously (*10*). The amount of *p*-nitroaniline released was measured by the absorbance at 410 nm, and the activity was calculated as units/mg protein. One unit is defined as the activity releasing 1 μmol of *p*-nitroaniline/min.

Thermal Stability of Subtilisin—To determine autolytic stability, the purified enzymes (20 μg/ml) were either treated with or without 2 mM dithiothreitol (DTT) at room temperature for 30 min. The remaining activity after heating for various times at 50–60°C in the presence of 1 mM $CaCl_2$ was determined at 37°C using *N*-succinyl-L-Ala-L-Ala-L-Pro-L-Phe-*p*-nitroanilide (AAPF) as the substrate.

Circular Dichroism (CD) Studies—CD measurements

were performed on a J-720 spectropolarimeter (Jasco, Tokyo) with a thermostatted cell holder controlled by an on-line temperature-control unit. The enzymes were dissolved in 10 mM sodium phosphate (pH 6.2) containing 1 mM $CaCl_2$ at a concentration of 0.14 mg/ml (5 μM). For the denaturation profiles, 5 μM enzymes and 0.01% (w/v) casein were dissolved in 50% (v/v) ethanol and 10 mM Tris-HCl (pH 8.5) containing 1 mM $CaCl_2$. For each spectrum, five scans were averaged from 260 to 200 nm at 4 or 25°C using a cell with a pathlength of 1 mm, with the wavelength increments set at 0.5 nm/s. All spectra were corrected by subtracting the spectrum of the buffer. The values are expressed as mean percents of CD change at 222 nm.

Molecular Modeling—Molecular modeling was performed with the computer programs Insight II and Homology (Molecular Simulations Inc., San Diego, USA). Modeling of subtilisin E was based on the X-ray crystal structure of subtilisin E with propeptide (PDB codes 1SCJ) (*25*).

RESULTS

Design of Disulfide Mutants—The amino acid residues of subtilisin E, Ser161 and Glu195, correspond to the Cys residues at positions 163 and 194, respectively, of aqualysin I when their sequences are aligned. However, in the mutant subtilisin containing Cys161 and Cys195, no disulfide bond was detected, probably because of the long distance between these two residues (*11*). In this study, taking into account the various criteria for a disulfide bond including the distance between the α-carbons of the residues, reasonable conformation, and a loop size (6 more residues) that is not in a tight turn and not part of an active site, four sites (Ser163/Ser194, Lys170/Ser194, Lys170/Glu195, and Pro172/Glu195) in subtilisin E were chosen as candidates for replacement with Cys residues by computational modeling (Table I). The positions chosen for the introduction of Cys residues were more than 14 Å away from the catalytic Ser221.

Expression of Mutant Subtilisins in E. coli—Nine mutant plasmids were constructed by site-directed mutagenesis to replace Ser163, Lys170, Pro172, Ser194, and Glu195 with Cys, and both Ser163 and Ser194, Lys170 and Ser194, Lys170 and Glu195, and Pro172 and Glu195 with Cys. The wild-type and mutant subtilisin E genes were expressed in *E. coli* JA221 in the presence of IPTG, and enzymes in the periplasmic fraction were assayed with a synthetic peptide substrate, AAPF, an authentic substrate for subtilisin. The relative activities of each mutant enzyme are shown in Fig. 1. The level of gene expression seems to be almost the same for all mutants, judging from the amounts of accumulated gene products in the periplasmic fraction as measured by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (data not shown). The single-Cys mutants at position 163, 170, 172, 194, and 195 showed significant decreases in enzymatic activity relative to the wild-type enzyme. When the double mutations S163C/S194C, K170C/S194C, and P172C/E195C were introduced, the catalytic activities were also severely impaired. It is noteworthy that the activity of the K170C/E195C mutant was approximately 70% that of the wild-type enzyme, whereas the single-Cys mutants at these positions (K170C and E195C) had less than 15% of the activity of the wild-type enzyme. Thus, we examined the properties of the K170C/E195C mutant subtilisin E further.

Expression of Disulfide Mutant Subtilisins in B. subtilis—Using the *E. coli* expression system, various mutant subtilisins were efficiently isolated and their enzymatic properties were examined (*10–14*). We could also detect prosubtilisin in the periplasmic fraction of *E. coli* cells, while the inactive-precursor was not released to the medium but accumulated in the *Bacillus* cell membrane (*20*). However, the *E. coli* expression system is disadvantageous from the view points of productivity and facility for use in the purification of recombinant subtilisin as compared to that of *B. subtilis*. We therefore constructed three plasmids, pIO-NSS, pIO-CSS, and pIO-DSS, carrying the mutant subtilisin E genes, G61C/S98C, K170C/E195C, and G61C/S98C/K170C/E195C, respectively, for the production in *B. subtilis* (Fig. 2). The wild-type and mutant subtilisin E genes were then expressed in *B. subtilis* DB403, and purified from the culture fluid to give a single band in SDS-PAGE. Generally, the formation of disulfide bond(s) can be detected by SDS-PAGE under nonreducing conditions because a protein that contains a disulfide bond has a smaller radius of gyration and therefore migrates further down the gel (*26*, *27*). Figure 3 shows that under nonreducing conditions, three mutant subtilisins (NSS for G61C/S98C, CSS for K170C/E195C, and DSS for G61C/S98C/K170C/E195C) did indeed migrate faster than the wild-type enzyme, whereas under reducing conditions the four bands were virtually indistinguishable. These results suggest that disulfide bonds

TABLE I. **Predicted distances between C_α atoms and between S_γ atoms in the various subtilisins E.**

Enzyme	Distances (Å)	
	C_α-C_α	S_γ-S_γ
G61C/S98C	5.3	4.3
S163C/S194C	7.0	6.0
K170C/S194C	8.7	8.7
K170C/E195C	5.4	3.4
P172C/E195C	7.0	5.0

The distances between residues were determined using the programs Insight II and Homology (Molecular Simulations Inc.).

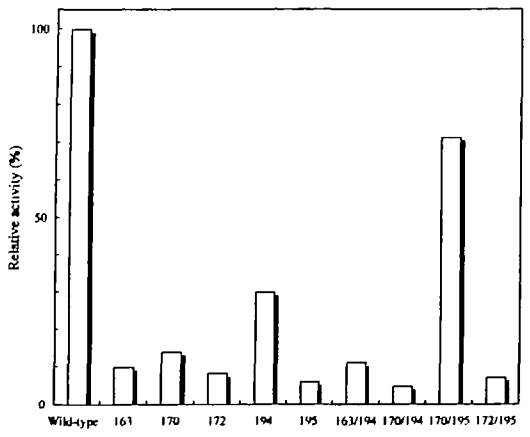

Fig. 1. **Protease activities of the wild-type and various mutant subtilisins E.** The periplasmic fractions were used as enzyme sources. Numbers indicate the position of the Cys substitution in each mutant subtilisin E. Enzyme asays were performed in 50 mM Tris-HCl (pH 8.5) and 1 mM $CaCl_2$ at 37°C using succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. Variations in the values were less than 5%.

formed spontaneously between Cys61 and Cys98, and between Cys170 and Cys195 in the *B. subtilis* expression system. In the case of the DSS mutant, the band was found to migrate faster than those of the NSS and CSS mutants, probably due to the formation of two disulfide bonds between Cys61/Cys98 and Cys170/Cys195. The evidence for disulfide formation in the double-Cys mutants was supported by assay according to the method of Ellman (*28*) using 5,5′-dithiobis (2-nitrobenzoic acid). No free sulfhydryl groups were detected in the double-Cys mutant or wild-type enzymes, while the single-Cys mutant at position 194 has free Cys residue (~1 μmol of Cys/μmol of protein).

Catalytic Properties of Disulfide Mutant Subtilisins—To examine the effect of disulfide bond(s) on catalysis, the kinetic constants of the purified subtilisins E variants were determined by AAPF (Table II). The catalytic efficiency of the NSS mutant was virtually unchanged from that of the wild-type enzyme, in agreement with previous results (*11*). On the other hand, it was found that the CSS and DSS mutants show a slight decrease in catalytic efficiency relative to the wild-type enzyme. The changes in k_{cat}/K_m were dominantly in the k_{cat} value, not in the K_m value. It should be noted that the k_{cat}/K_m values of all the enzymes were essentially equivalent, regardless of whether they were pretreated with 2 mM DTT for reduction (Table II). These results suggest that Cys substitutions at positions 170 and 195 might affect the local structure around the active site.

Our previous work showed that a Leu substitution at position 31 (Ile in the wild-type subtilisin E), which is adjacent to the catalytic site Asp32, results in a remarkable increase in specific activity due to a larger k_{cat} for all substrates (*10*). To recover the specific activity of the DSS mutant, we constructed the quintet mutant LDSS (I31L/G61C/S98C/K170C/E195C) as described in the legend to Fig. 2. As shown in Table II, the LDSS mutant showed a 2-fold increase in catalytic efficiency due to a large k_{cat}, in agreement with previous results (*10*). We did not examine the specific activity for a natural protein substrate such as casein. However, judging from our previous findings (*10, 11*), results similar those obtained using synthetic peptide substrates seem likely.

When assayed using synthetic peptide substrates, *N*-succinyl-L-Ala-L-Ala-L-Pro-L-X-*p*-nitroanilide (X = Ala, Glu, Leu, Lys, Met, Phe, and Val), or oxidized insulin A- and B-chains (Sigma), the substrate specificities of the mutants were similar to those of the wild-type enzyme (data not

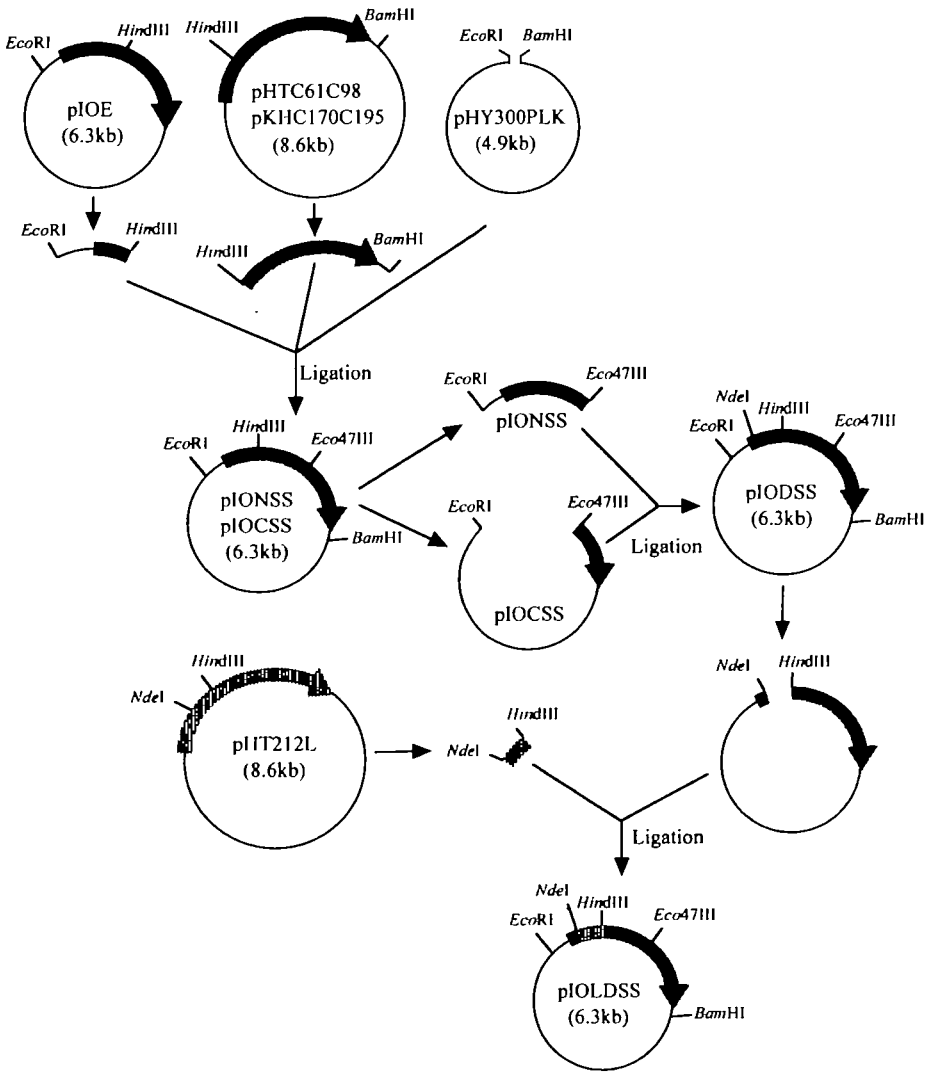

Fig. 2. **Construction of expression plasmids for subtilisin E genes in *B. subtilis*.** Using a plasmid pHI212 harboring the wild-type subtilisin E (*10*), plasmids with substitutions (G61C/S98C and K170C/E195C) were constructed by site-directed mutagenesis and named pHTC61C98 (*11*) and pKHC170C195 (this study), respectively. The 0.8 kb *Hin*dIII–*Bam*HI fragment of plasmids pHTC61C98 and pKHC170C195, the 0.6 kb *Eco*RI–*Hin*dIII fragment of pIOE, and the 4.9 kb *Eco*RI–*Bam*HI fragment of pHY300PLK were mixed, ligated, and introduced into *E. coli* C600 to construct two mutant plasmids, pIONSS and pIOCSS, respectively. The 0.9 kb *Eco*RI–*Eco*47III fragment of pIONSS was then ligated to the large fragment of pIOCSS digested with *Eco*RI and *Eco*47III. The resultant plasmid (pIODSS) contains the quadruple mutations G61C/S98C/K170C/E195C. Further, the 180 bp *Nde*I–*Hin*dIII fragment of plasmid pHT212L with the I31L mutation in subtilisin E (*10*) was ligated to the large fragment of pIODSS digested with *Nde*I and *Hin*dIII to construct the plasmid pIOLDSS, including the quintet mutations I31L/G61C/S98C/K170C/E195C. These plasmids were then introduced into *B. subtilis* DB403 by electroporation. The arrows in the plasmids represent the subtilisin E gene containing the original promoter and terminator regions.

shown). The pH profiles of the wild-type and mutant subtilisins were also measured with AAPF. All the enzymes showed optima at pH 8.5–9.0 and were stable in the pH range 6.0–10.0 (data not shown).

Thermal Stability of Disulfide Mutant Subtilisins—It has been shown for some serine proteases that reduced autolysis leads to an apparent increase in stability (*29*). To determine the autolytic stability of these enzymes, the rate of thermal inactivation was measured at 55°C in the presence of Ca^{2+}. The half-life ($t_{1/2}$) of the CSS mutant was 60% that of the wild-type enzyme (Table III). It is considered that the disulfide bond might cause an unfavorable structural change in subtilisin; however, a remarkable drop in stability was observed in the reduced mutant when the enzyme was treated with 2 mM DTT. The stability of the DSS mutant was nearly equal to that of the CSS mutant, whereas the $t_{1/2}$ of the NSS mutant was found to be 2–3 times longer than that of the wild-type enzyme, as described previously (*11*). A similar tendency was still observed at 50 and 60°C (data not shown). These results suggest that the ionic interaction between Lys170 and Glu195 is important for structural stability in the wild-type enzyme, and that the corresponding disulfide bridge also contributes to the over-all thermal stability.

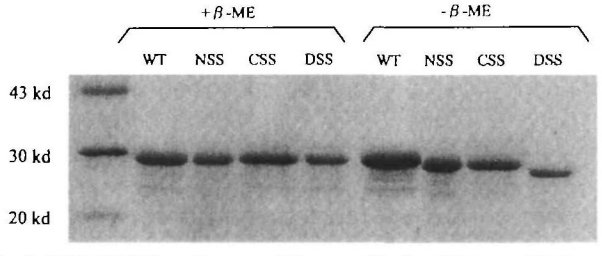

Fig. 3. **SDS-PAGE patterns of the purified wild-type (WT) and disulfide mutant (NSS, CSS, and DSS) subtilisins E.** Purified enzymes were inactivated with phenylmethanesulfonyl fluoride before boiling in SDS solution to limit the autolysis that accompanies denaturation. For samples in the four left lanes, the SDS solution also contained 1 mM β-mercaptoethanol (β-ME) as a reducing agent. β-ME was omitted from the non-reduced samples in the other four lanes. Three micrograms of protein was loaded in each lane. SDS-PAGE was performed in a 15% polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue. Molecular mass standards are shown at the left.

TABLE II. **Kinetic constants of the wild-type and disulfide mutant subtilisins E for the hydrolysis of succinyl-Ala-Ala-Pro-*p*-nitroanilide.**

Enzyme	DTT	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m (s^{-1} mM^{-1})
Wild-type	−	20.4±2.0	1.68±0.3	12.2±1.3
	+	19.9±2.0	1.52±0.1	13.1±0.8
NSS	−	26.0±3.8	1.65±0.2	15.9±2.6
	+	25.3±1.5	1.64±0.1	15.4±0.7
CSS	−	9.01±1.4	1.51±0.1	5.97±0.5
	+	8.01±0.5	1.38±0.1	5.80±0.6
DSS	−	17.2±1.1	1.99±0.1	8.69±0.4
	+	16.3±2.1	1.87±0.4	8.72±0.4
LDSS	−	30.0±1.2	1.76±0.1	17.0±1.0
	+	29.2±2.2	1.72±0.2	17.0±2.2

Purified enzymes were treated with or without 2 mM DTT at room temperature for 30 min. Assays were carried out in 50 mM Tris-HCl (pH 8.5) and 1 mM $CaCl_2$ at 37°C. The data shown are means ± standard deviations of three independent experiments.

Similar results were also obtained by differential scanning calorimetry (DSC). The CSS mutant showed a lower T_m (midpoint in the thermally induced transition from the folded to the unfolded state) than that of the wild-type enzyme. However, after treatment with phenylmethanesulfonyl fluoride, a serine protease inhibitor, to prevent the autolysis that accompanies the unfolding of the enzyme, no reliable DSC scan could be observed at 60°C or above for any the samples except the wild-type subtilisin due mainly to the aggregation of the denatured proteins (data not shown).

Stability of Disulfide Mutant Subtilisins in Polar Organic Solvents—We further examined the stabilities of the disulfide enzymes in non-natural environments. In the presence of protein denaturants, including SDS, urea, and guanidine hydrochloride, the mutant subtilisins were found to be slightly less stable than the wild-type enzyme (data not shown).

One noteworthy observation was seen in polar organic solvents. When the concentration (v/v) of dimethylformamide (DMF) and ethanol that inhibit subtilisin activity by 50% (IC_{50}) were determined for each enzyme, the CSS and DSS mutants showed an approximately 1.5-fold increase in IC_{50}, indicating that these enzymes are more stable than the wild-type in DMF and ethanol (Table IV). However, even under reducing conditions (+DTT), the stability of

TABLE III. **Thermal stability of the wild-type and disulfide mutant subtilisins E.**

Enzyme	DTT	Half-life at 55°C (min)
Wild-type	−	26.2
	+	24.1
NSS	−	81.0
	+	32.0
CSS	−	15.9
	+	3.8
DSS	−	16.9
	+	8.3

Purified enzymes were treated with or without 2 mM DTT at room temperature for 30 min. Remaining activity was determined at 37°C using succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. Half-life due to autolytic inactivation was determined from the semi-log plot of $\log_{10}$ [residual activity] *versus* time. The variations in the values were less than 5%.

TABLE IV. **Stability of the wild-type and disulfide mutant subtilisins E in polar organic solvents (DMF and ethanol).**

Enzyme	DTT	IC_{50} (%)	
		DMF	Ethanol
Wild-type	−	9.0	7.5
	+	9.2	NT
NSS	−	9.0	7.5
	+	9.1	NT
CSS	−	13.1	11.6
	+	13.2	NT
DSS	−	14.7	12.3
	+	14.8	NT
LDSS	−	16.4	11.6
	+	16.5	NT

Purified enzymes were treated with or without 2 mM DTT at room temperature for 30 min. The concentration of DMF and ethanol that inhibits 50% subtilisin activity (IC_{50}) was determined at 37°C in the presence of $CaCl_2$ using succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide. The variations in the values were less than 5%. NT, not treated.

both mutants was essentially equivalent to that of the disulfied forms. These results suggest that the disulfide bond between Cys170 and Cys195 has little influence on the stability of the enzyme in organic media and that the stabilization is probably due to the substitution of the uncharged Cys residue for the charged surface residues (Lys170 and Glu195).

The CD spectra of the wild-type and mutant enzymes were measured at 4°C to determine whether the introduced disulfide bond(s) affects the secondary structure of subtilisin (Fig. 4A). No differences were observed, suggesting that the disulfide bonds do not cause any change in the secondary structure. The denaturation profiles in the presence of 0.01% (w/v) casein and 50% (v/v) ethanol were also analyzed by measuring the change in ellipticity at 222 nm (Fig. 4B). Figure 4B shows that the CSS, DSS, and LDSS mutant subtilisins retained their tertiary structures and were more stable than the wild-type or NSS mutant subtilisins against denaturation by ethanol.

Enhanced Activity of Disulfide Mutant Subtilisins in DMF—The kinetic constants of the purified subtilisin E variants were determined from the initial rate measurements for the hydrolysis of AAPF in DMF concentrations up to 40% (v/v). As shown in Fig. 5, it was impossible to obtain the individual kinetic parameters for the wild-type and NSS mutant subtilisins in high concentrations of DMF [above 20% (v/v)], owing to the marked increase in K_m, which indicates little affinity of the enzymes for the substrate. In contrast, the CSS and DSS mutant subtilisins were more active than the wild-type or NSS mutant enzymes. When Ile31 was replaced by Leu in the DSS mutant (LDSS), a significant increase in catalytic efficiency occurred, ranging from 2–4-fold for AAPF, relative to the CSS and DSS mutants at any concentration tested (Fig. 5).

Molecular Modeling in the DSS Mutant Subtilisin—

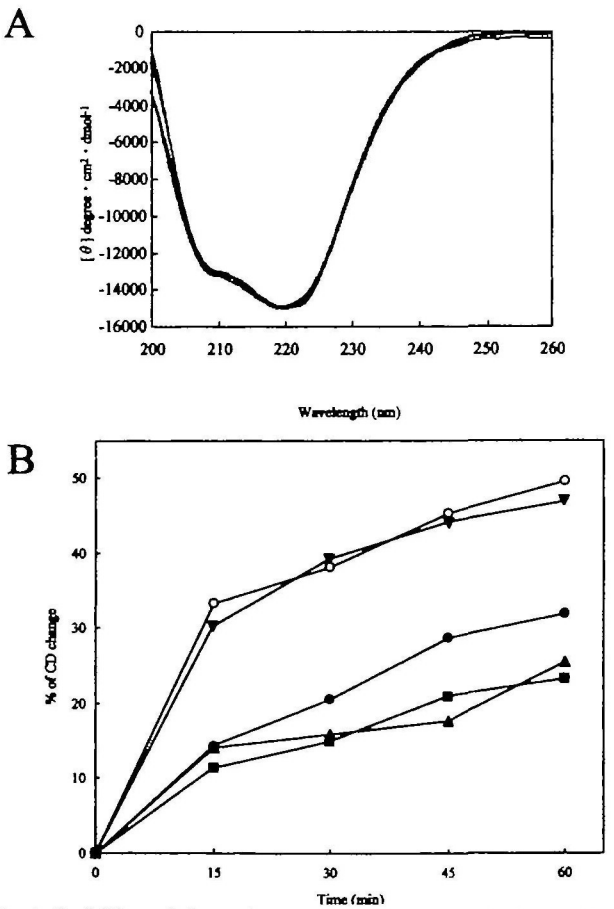

Fig. 4. **Stability of the wild-type and mutant subtilisins E in polar organic solvents.** (A) CD spectrum of the wild-type (black line), NSS (red line), CSS (blue line), DSS (orange line), and LDSS (green line) mutant subtilisins E in 10 mM sodium phosphate (pH 6.2), 1 mM $CaCl_2$. For each spectrum, fifteen scans were averaged over the absorption range 260 to 200 nm at 4°C. [θ] stands for mean residue ellipticity. (B) The denaturation profiles of the wild-type (○), NSS (▼), CSS (●), DSS (▲), and LDSS (■) mutant subtilisins E in ethanol. 50% ethanol-induced denaturation was monitored by the changes in CD at 222 nm. For each spectrum, five scans were averaged over the absorption range 260 to 200 nm at 25°C. Variations in the values were less than 5%.

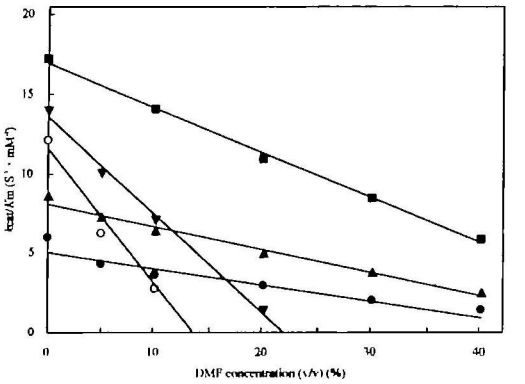

Fig. 5. **Catalytic efficiency of the wild-type (○), NSS (▼), CSS (●), DSS (▲), and LDSS (■) mutant subtilisins E in various concentrations of DMF (v/v).** Kinetic constants k_{cat} and K_m were determined from initial rate measurements for the hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide in 50 mM Tris-HCl (pH 8.5) and 1 mM $CaCl_2$ at 37°C. Variations in the values were less than 5%.

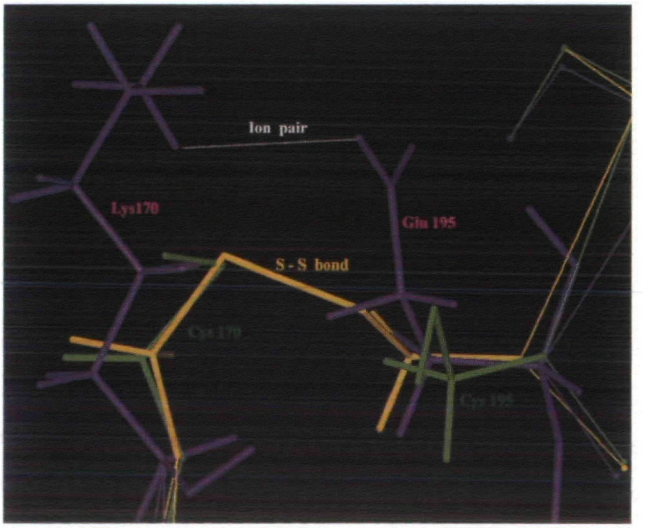

Fig. 6. **Computer modeling of the α-carbon backbone structure of subtilisin E.** The wild-type (Lys170/Glu195) and Cys170/Cys195 mutant subtilisins E were drawn using the programs Insight II and Homology (Molecular Simulations Inc.)

Computational modeling was conducted to predict the structure around the Cys substitution sites (Fig. 6). Based on the X-ray analysis of subtilisin E (*25*), it is probable that there is an electrostatic interaction between Lys170 and Glu195 that is important for the catalysis and structural stability of the wild-type enzyme. The role of the ionic interaction would be partially compensated for by the introduced disulfide bond. On the other hand, the charged surface residues were replaced by uncharged Cys, and the substitutions might stabilize subtilisin E in polar organic solvents.

DISCUSSION

So far, only our engineered disulfide bond, which was designed with reference to the disulfide bond in aqualysin I, causes a considerable enhancement in the thermal stability of subtilisin E (*11*). X-ray crystallographic data for subtilisin BPN′ (*30*) and subtilisin E (*25*) show that residues 61 and 98 are not located in α-helix, β-sheet or a tight turn. Such positions in subtilisin E could produce a good geometric fit for a disulfide bond. Recently, Mansfeld *et al.* (*31*) reported that a *de novo* disulfide bond had been designed in a surface-located region in the N-terminal domain of the thermolysin-like protease produced by *B. stearothermophilus*, and this resulted in a drastic stabilization of the enzyme. One can still conclude that naturally occurring disulfide bridges should provide a better model for the stabilization of subtilisins than previously designed ones. Siezen *et al.* reported that seven naturally occurring disulfide bonds have been identified in subtilases, enzymes that encompass over 40 members of the subtilisin-like serine protease family (*32*, *33*). However, extracellular enzymes in Gram-positive bacteria, such as subtilisins, rarely contain disulfides. Bridges that are unlikely to provide significant extra stability are found only in subtilisins TA41 and S39 from psychophilic *Bacillus* sp. (*34*, *35*). On the other hand, the two disulfide bonds in aqualysin I seem to be responsible for the heat stability of that enzyme (*17*).

In the present study, it is evident that the sites for Cys substitution are crucial for both the thermostability and catalysis of the enzyme. Only the DSS mutant showed a marked increase in catalytic activity compared with the single-Cys mutants (Fig. 1), and was greatly stabilized relative to its reduced form (Table III). However, the catalytic efficiency of the DSS mutant was unchanged even after DTT treatment (Table II). These findings suggest that the role of an electrostatic interaction between Lys170 and Glu195 in the stability of subtilisin can be compensated for to some extent by the introduced disulfide bond. Considering the roles of Lys170 and Glu195, it is reasonable to assume that the activity of the K170C/S194C and P172C/E195C mutants would be severely impaired because the counterpart that forms the electrostatic interaction is replaced by Cys. However, the Cys170/Cys195 disulfide mutant was found to be thermolabile relative to the wild-type. The combination of two disulfide bonds (Cys61/Cys98 and Cys170/ Cys195) in the DSS mutant also did not lead to thermostability, whereas the N-terminal disulfide bond (Cys61/Cys98) enhanced the stability without any change in catalytic activity (*11*). Further studies of the function of subtilisin E makes it worthwhile to design another disulfide bond variant around these positions. In addition, the Cys163/Cys194 mutant subtilisin also appears to form a disulfide bond in the *B. subtilis* expression system. However, the disulfide bond causes a marked decrease in stability and catalytic activity probably due to structural disruption (data not shown). The question arises as to why Cys replacements at positions 163, 172, and 194 cause a massive decrease in catalytic activity. Although the role of these residues in subtilisin is poorly understood, one possibility is that a drastic change in the distance between Lys170 and Glu195 may weaken the interaction.

One interesting finding concerns the stability of the mutant subtilisins in polar organic solvents. In the presence of DMF and ethanol, the CSS and DSS mutants were stabilized with respect to the wild-type and the NSS mutant enzymes (Table IV). The CD profiles of the subtilisin variants in the presence of casein as a substrate, which causes an induced-fit of the enzyme, confirmed the stabilization of these mutants (Fig. 4). In general, in order to stabilize enzymes in organic solvents, (i) substitution of selected charged surface residues by uncharged amino acids, and (ii) an improvement in internal hydrogen bonds is necessary (*36*). In the case of subtilisin E, a charged surface residue, Asp248, was replaced by three amino acids of increasing hydrophobicity, Asn, Ala, and Leu. The Asn218Ser mutation, isolated as a thermostable variant (*37*), showed altered internal hydrogen bonding interactions and stabilized subtilisin E in high concentrations of DMF (40%) (*36*). Recently, multiple site-directed mutagenesis or sequential rounds of random mutagenesis have been used to engineer subtilisins BPN′ and E to function in high concentrations of DMF (*38–40*). The modeling analysis showed that the cumulative amino acid substitutions in the variant are positioned in variable loops on the surface of the enzyme surrounding the active site (*40*). In contrast, the K170C/E195C mutant subtilisin E in the present study is of great interest in terms of its stabilization in polar organic solvents despite its lowered thermostability. The substitution of uncharged Cys for charged surface residues (Lys170 and Glu195) in a variable loop would mainly facilitate the stabilization of the enzyme in polar organic solvents, not stabilization to thermal autolysis. The increased stability in polar organic solvents was considered to be due to the replacements of charged residues rather than to the introduction of the disulfide bond (Table IV). Uncharged amino acids other than Cys may also contribute to the stability in polar organic solvents; however, a significant decrease in catalytic efficiency occurs even in aqueous solution when residues 170 and 195 are replaced with another uncharged residue, Ala (data not shown). Therefore, the disulfide bond leads to the retention of the catalytic activity and the overall stability in polar organic solvents. Structural analysis of mutant subtilisins in polar organic solvents would serve to elucidate the mechanism.

Subtilisin is a useful catalyst for organic synthesis, particularly in the presence of organic solvents, and it also catalyzes peptide synthesis (*38*). Unfortunately, the enzyme stability and activity are often drastically reduced in water-miscible organic solvents such as DMF and ethanol. Even though we succeeded in stabilizing subtilisin E in polar organic solvents, the hydrolyzing activity of the mutant enzyme was considerably lower than that of the wild-type. The low protease activity would not be useful for industrial application. To recover catalytic efficiency, Leu was replaced

by Ile31, a change that leads to a remarkable increase in specific activity due to a larger k_{cat}. Residue 31 in subtilisin supposedly serves to stabilize Asp32, which accepts the His64 proton. We now speculate that a slight change in the distance between Asp32 and His64 in the wild-type and Leu31 mutant may occur, thus facilitating proton transfer.

In conclusion, we constructed a novel mutant subtilisin E with high stability and activity in polar organic solvents through multiple amino acid substitutions, and attempts to achieve peptide synthesis with the variant enzyme is currently in progress.

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