Expression of *Bacillus* Protease (Protease BYA) from *Bacillus* sp. Y in *Bacillus subtilis* and Enhancement of Its Specific Activity by Site-Directed Mutagenesis—Improvement in Productivity of Detergent Enzyme—

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An attempt was made to express protease BYA produced by an alkalophilic *Bacillus* sp. Y in *Bacillus subtilis* by gene engineering methods. The gene encoding protease BYA was cloned from *Bacillus* sp. Y, and expression vector pTA71 was constructed from the amylase promoter of *Bacillus licheniformis*, DNA fragments encoding the open reading frame of protease BYA, and pUB110. Protease BYA was secreted at an activity level of 5100 APU/ml in the common industrial culture medium of *Bacillus subtilis* transformed with pTA71. We then attempted to increase the specific activity of protease BYA by site-directed mutagenesis. Amino acid residue Ala29 next to catalytic Asp30 was replaced by one of three uncharged amino acid residues (Val29, Leu29, Ile29), and each mutant enzyme was expressed and isolated from the culture medium. Val29 mutant enzyme was secreted at an activity level of greater than 7000 APU/ml in culture medium, and its specific activity was 1.5-fold higher than that of the wild-type enzyme. Other mutant enzymes had specific activity similar to that of the original one and were less stable than the wild-type enzyme. It can be thought that the substitution at amino acid residue 29 affects the level of activity and stability of protease BYA.

Key words protease BYA; subtilisin; expression; *Bacillus*; bacteriolysis; detergent protease

Subtilisins produced by genus *Bacillus* belong to the general class of serine proteases, they all share the active site (Ser, His, and Asp, respectively).¹⁾ Some subtilisins, represented by subtilisin Carlsberg from *Bacillus licheniformis*, are added to detergents products to remove soil effectively.^{1–4)} They are required to be stable in detergent solutions containing alkali and surfactants, and stable under high-temperature washing conditions.⁵⁾ Shimogaki *et al.* isolated a subtilisin called *Bacillus* protease BYA produced by an alkalophilic strain designated *Bacillus* sp. Y (FERM-8088).⁶⁾ It retains 90% of its protease activity after 30 min in 0.1 N NaOH solution, 80% after 4 h in sodium linear alkylbenzene sulfonate solution, and 90% after 10 min at 60 °C, whereas subtilisin Carlsberg completely loses its activity under the same conditions. Additionally, protease BYA shows bacteriolytic activity.⁷⁾ It lysed gram-negative bacteria representing *Pseudomonas aeruginosa*, and some gram-positive bacteria like *Staphylococcus aureus* well, whereas subtilisin Carlsberg hardly lysed them. Bacteriolytic activity of protease BYA is superior to that of Deozyme,⁸⁾ one of subtilisin known as superior bacteriolytic enzyme, in out of 40 °C. It is believed that protease BYA contributes to improvement of sanitation by its detergency and bacteriolytic activity. From the standpoint of application, it is important to express it by gene engineering. In this study, we attempted to clone protease BYA gene, express it in *Bacillus subtilis* by molecular cloning, and enhance the specific activity of protease BYA by site-directed mutagenesis.

MATERIALS AND METHODS

Strains *Bacillus* sp. Y (FERM-8088), *Bacillus licheniformis* ATCC14580, and *Escherichia* (*E.*) *coli* HB101, JM109, GM2163 were used for gene cloning. *Bacillus subtilis* 1012 (FERM-7168), a variant of *Bacillus subtilis* Marburg 168 that secretes a little of its protease, subtilisin E, by mutation, was used as a host for expression of protease BYA.

DNA Probes and Primers DNA probes and primers shown in Table 1 were synthesized with a DNA synthesizer (Applied Bio System 381A). The DNA probes (a) and (b) were designed based on the amino acid residues at the N-terminal (Asn-Pro-Val-Ala-Arg-Gly-Ile-Val-Lys-Ala-Asp-Val-Ala-Gln-Asn-Asn-Tyr-Gly-Leu-Tyr-Gly)⁶⁾ and one of the lysylendopeptidase digestion peptides (Lys-Tyr-Ala-Tyr-Met-Gly-Gly-Thr-Ser-Met-Ala-Thr) that containing the common amino acid sequence around the active site of subtilisin. They were synthesized by the deoxyinosine method⁹⁾ with gamma 32P-ATP (Amersham).

Cloning of Protease BYA Gene Most of the methods used for molecular cloning were based on those of Maniatis *et al.*¹⁰⁾ *Bacillus* sp. Y was grown aerobically at 30 °C overnight in nutrient broth (Kyokutouseiyaku) containing Na₂CO₃ 10 g/l. Chromosomal DNA was prepared essentially as described by Saito and Miura.¹¹⁾ After digestion with restriction enzymes, the fragments were separated by agarose gel electrophoresis. The gel was dehydrated, and agarose-membrane hybridization was performed using each of the DNA probes. DNA fragments that hybridized to each DNA probe were separated by agarose gel electrophoresis and col-

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Fig. 1. Structure of Each Plasmid

The open arrow represents the deduced promoter region of the protease BYA gene. The solid arrows represent the promoter region of the *Bacillus licheniformis* amylase gene (Amy). The striped arrows represent the open reading frame of the protease BYA gene (ORF), E: *Eco* RI site; B: *Bcl* I site; S: *Sph* I site.

lected by the DEAE paper method.¹⁰⁾ Each DNA fragment was ligated to plasmid vector pBR328 or pUC118 treated with each restriction enzyme and alkaline phosphatase, and the ligated DNA was used to transform *E. coli*. Plasmids extracted from transformants were hybridized with the use of each probe according to the Southern hybridization method.¹⁰⁾

Cloning of Amylase Promoter Gene *Bacillus licheniformis* was grown aerobically at 30 °C overnight in nutrient broth (Kyokutouseiyaku). Chromosomal DNA was prepared essentially as described by Saito and Miura.¹¹⁾ After digestion with *Eco* RI, these fragments were ligated to pUC118 treated with *Eco* RI and alkaline phosphatase. *E. coli* transformed with the ligated DNA was incubated on Luria agar containing 10 µg/ml of ampicillin and 1% potato starch, and the colonies were examined for a halo of digested potato starch.

Construction of Expression Vectors Plasmids to express protease BYA were constructed as shown in Fig. 1. pUY31 was constructed from approximately 4.8 kbp DNA fragment encoding the protease BYA gene, pBR328, and pUB110.¹²⁾ The *Bcl* I site (TGATCA) was created at the 1014 bp of amylase gene shown in Figs. 2 and 3 with primer (c) shown in Table 1 by site-directed mutagenesis. The *Bcl* I sites were also created in 200–216 bp region of the protease BYA gene with primer (d), (e) and (f) as shown in Figs. 3 and 4. pTA52A was constructed from pBR328, pUB110, and DNA fragments created with primer (c), (d). pTA52B and pTA52C were also constructed as well as pTA52A with primer (e) and (f) shown in Fig. 3. To do so efficiently, the

Bcl I site at 223 bp upstream of the amylase promoter shown in Fig. 2 was deleted with primer (g) by site-directed mutagenesis, and the *Eco* RI site (GAATTC) of the protease BYA gene, at 1190 bp shown in Fig. 4, was also deleted with primer (h), which made no alternation in the amino acid sequence of protease BYA. pTA71 was derived from shortening of pTA52A.

DNA Sequencing and Site-Directed Mutagenesis The restriction fragments to be sequenced were subcloned into pUC118 or pUC119, and ssDNA of each fragment was prepared using M13K07 phage.¹³⁾ DNA sequencing by the chain-termination method was performed according to Sanger.¹⁴⁾ Both strands of the region analyzed were sequenced. The amino acid sequences of protease BYA and other proteases were aligned with DDBJ.¹⁵⁾ Site-directed mutagenesis was carried out according to the method of Kunkel *et al.*,¹⁶⁾ using a Muta Gene *in vitro* mutagenesis kit (Bio-Rad). Each mutation was confirmed by DNA sequence analysis according to Sanger.¹⁴⁾

Expression of Proteases and Enzymatic Analysis *Bacillus subtilis* 1012 was transformed as described by Chain.¹⁷⁾ *Bacillus subtilis* 1012 transformed with a plasmid vector was grown aerobically at 33 °C for approximately 90 h in common industrial culture medium containing 20 g of soluble starch, 20 g of polypepton S (Daigoeiyu), 5 g of K₂HPO₄, 0.2 g of MgSO₄·7H₂O, 50 mg of kanamycin sulfate per liter, pH 7.5. Protease BYA and mutant enzymes secreted into the culture medium were purified, and their specific activity was measured as described Shimogaki *et al.*⁶⁾ The amount of enzyme that liberated 1 µg of tyrosine per min

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10      20      30      40      50      60      70      80      90      100     110     120
GAATTCAGATTTTCTGAACAGGATTTCAAGCATTGCCTATCAGGAATTGAAAAATCGGAATCCGCTCTGCTGACATGGCTTTATTTAAAGAAGGGACGAGCCCTCAGGAAGAACATTTTCAG
EcoRI
130     140     150     160     170     180     190     200     210     220     230     240
CCTTCGCCCTTCTTGAAAACGAGGTGGAATTTTATTTTGAGAAAAACAAACCGGTCTTGAAACAGTCTGTAAAAATAAAAAAGAATGGGTAAAAGATATTTTAAAGACCGATATCAGGA
250     260     270     280     290     300     310     320     330     340     350     360
TATGAAAAAGAAATCGTCTTCAGGCCAAACCTGATCAGGAGCCTGTTCCGCTTCCGAAGCAAGCGAAAATTAATCCCGATGAAAAAGTGATTGCCCTCACATTTGATGACGGTCCGAATCC
BclI
370     380     390     400     410     420     430     440     450     460     470     480
CGCTACAACGAATAAAATATTAACGCTTTACAGAAGCATGAAGGGCATGCGACCTTCTTTGTGCTTGAAGCAGAGCGCAATATTATCCCGAAACGATAAAACGGATGCTGAAGGAAGG
490     500     510     520     530     540     550     560     570     580     590     600
AAACGAAGTCGGCAACCATTCCTGGGACCATCCGTATTGACAAGGCTGTCAAACGAAAAAGCGTATCAGGAGATTAACGACACGCAAGAAATGATCGAAAAAATCAGCGGACACCTGCC
610     620     630     640     650     660     670     680     690     700     710     720
TGTAACCTTGCGTCTCCATACGGCGGGATCAATGATTCCGTCGCTTCCAATCTGAAGGTTTCATTGTGGGATGTTGATCCGGAAGATTGGAAGTACAAAAATAAGCAAAAGAT
730     740     750     760     770     780     790     800     810     820     830     840
TGTAATCATGTGATGAGCCATGCGGAGACGGAAAAATCGTCTTAATGCACGATATTTATGCAACGTCGCAGATGCTGCTGAAGAGATTATTAAGAGCTGAAAGCAAAAGGCTATCAA
850     860     870     880     890     900     910     920     930     940     950     960
TTGGTAACTGTATCTCAGCTTGAAGAAGTGAAGAAGCAGAGAGGCTATTGAATAAATGAGTAGAAGCGCCATATCGCGCTTTTCTTTTGAAGAAAAATAGGAAAAATGGTATTGTGT
970     980     990     1000    1010    1020    1030    1040    1050    1060    1070    1080
AAAAATTCTGAATATTTATACAATATCATATGTTTCACATTGAAAGGGGAGGAGAATCATGAAACAACAAAAACGGCTTTACGCCGATTGCTGCCGCTGTTATTATGCGCTCATCTTCTT
*****  ***  **      M  K  Q  Q  K  R  L  Y  A  R  L  L  P  L  L  F  A  L  I  F
SD

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Fig. 2. Nucleotide Sequence of the Amylase Gene of *Bacillus licheniformis*
The putative Shine-Dalgarno sequence (SD) is indicated by the stars. Amino acids are represented by their single-letter code.

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Amylase gene original; -TTTCACATTGAAAGGGGAGGAGAATCATGAAACAACAAAAACGGCTTT-
                      *****  ***  **

pTA52A; -TTTCACATTGAAAGGGGAGGATGATCAATGAAGGGGAAAAAAGAGTA-
pTA52B; -TTTCACATTGAAAGGGGAGGATGATCAGAAATGAAGGGGAAAAAAGA-
pTA52C; -TTTCACATTGAAAGGGGAGGATGATCAGTTGAGGAGAAATGAAGGGGA-

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Fig. 3. Construction of *Bcl* I Sites in the Amylase Gene and the Protease BYA Gene
The putative Shine-Dalgarno sequence (SD) is indicated by stars. Initial codons are underlined. DNA fragments of the protease BYA gene are in bold print.

Table 1. DNA Probes and Primers Designed for Cloning and Site-Directed Mutagenesis

(a) 5'-CCATA(A/G)TT(A/G)TT(T/C)TGIGCIACATCIGC-3'
(b) 5'-GTICCCCATATAIGC(A/G)TA(T/C)TT-3'
(c) 5'-TGTGTTTCATGATCAICCTCCCCTTTCAA-3'
(d) 5'-TTTCCCTTCATTGATCATCAACTCCTCAT-3'
(e) 5'-TTTCCCTTCATTCTGATCAACTCCTCAT-3'
(f) 5'-TTTCTCCTCAACTGATCATCTTCATTCATT-3'
(g) 5'-GGAACAGGCTCCTGGTCAGGTTTGGCCTGA-3'
(h) 5'-CCAAGAGTAGTAGTGGATTCTTGCTCCAGC-3'
(i) 5'-CTAGTTGCAGTAGTTGACACAGGCTTAGAT-3'
(j) 5'-AACTAGTTGCAGTACTTGACACAGGCTTAG-3'
(k) 5'-CTAGTTGCAGTAATTGACACAGGCTTAGAT-3'

Substitutions of the original nucleotides sequence are underlined.

under the condition described by Shimogaki *et al.*⁶⁾ was defined as one unit of alkaline protease activity (1 APU). The heat-, alkali-, and surfactant-stability of the proteases was evaluated according to Shimogaki *et al.*⁶⁾ Each protease (1000 APU/ml) was incubated under various conditions.

RESULTS

Cloning of Protease BYA Gene Several DNA probes were designed, and two DNA probes (a) and (b) that clearly hybridized to chromosomal DNA were used for cloning.

DNA probe (a) always hybridized to an approximately 2.8 kb *Eco* RI or 1.2 kb *Xba* I fragment. DNA probe (b) always hybridized to an approximately 2.0 kb *Eco* RI or 1.2 kb *Xba* I fragment. Each DNA fragment was collected and ligated to plasmid vector pBR328 or pUC118, and the ligated DNA was used to transform *E. coli*. Each plasmid of 500 transformants was prepared and hybridized by the Southern hybridization method with each of the probes. A few plasmids from each transformant reacted strongly with DNA probe (a) or (b). Plasmids containing each fragment to which the labeled probes hybridized were detected and named pYT101 (2.8 kb, *Eco* RI), pYB002 (2.0 kb, *Eco* RI), and pYX001 (1.2 kb, *Xba* I). The results of sequence analysis of clones and the deduced amino acid sequence are shown in Fig. 4. The 21 N-terminal residues and 12 residues in the vicinity of the active site identical with those of mature protease BYA are underlined. The open reading frame of the protease BYA gene comprised 1905 nucleotides. The deduced amino acid sequence indicated that protease BYA consists of 635 amino acid residues and has a molecular mass of 67678 Da. The mature protease BYA consists of 433 amino acid residues (between +1 to +433), and has a molecular mass of 45573 Da.

Cloning of Amylase Promoter A transformant that formed a halo was obtained from approximately 2000 colonies in a chromosome library of *Bacillus licheniformis* in

Fig. 4. Nucleotide Sequence of the Protease BYA Gene, and the Deduced Amino Acid Sequence of the Protein

A putative Shine-Dalgarno sequence (SD) and a transcriptional termination signal (arrows) are indicated. Amino acids are represented by their single-letter code. The open reading frame extends from Met (−202) to His (+433). The 21 N-terminal residues (+1—+21) and 12 residues in the vicinity of the active site (+246—+257) identical with those of mature protease BYA are underlined.

E. coli. Plasmid (pTA01) including an approximately 3.5 kbp *Eco* RI fragment was extracted from the transformant. The sequence in the vicinity of the amylase promoter in pTA01 was determined and is shown in Fig. 2. The sequence was found to have 99% homology with the sequence of the amy-

lase promoter reported by Yuuki *et al.*¹⁸⁾

Expression of Protease BYA *Bacillus subtilis* 1012 was transformed with pUB110, pUY31, pTA52A, pTA52B, pTA52C, or pTA71, and incubated in culture medium. Shown as Table 2, a transformant carrying pUB110 secreted

Table 2. The Amounts of Protease BYA Produced by Transformants Carrying Various Recombinant Plasmids

Plasmids	APU/ml
pUB110	320
pUY31	370
pTA52A	3400
pTA71	5100
pTA71 (Val29)	7200

Alkaline protease units (APU/ml) were measured⁽⁶⁾ in the culture medium of *Bacillus subtilis* 1012 carrying each plasmid.

Fig. 5. Specific Activity of Protease BYA Mutants (Val29, Leu29, Ile29) in Comparison with Protease BYA (Ala29)

The concentrations of each enzyme were made uniform. Their activity was measured with casein or keratin as the substrate, and their specific activity was evaluated by comparison with Ala29.

320 APU/ml of alkaline protease in the culture medium, and a transformant carrying pUY31 secreted 370 APU/ml. On the other hand, 3400 APU/ml of alkaline protease was secreted by a pTA52A transformant, and 5100 APU/ml of alkaline protease was secreted by a pTA71 transformant. The secreted protein was purified by Shimogaki's method,⁽⁶⁾ and detected as single band which has approximately 45000 Da by SDS-PAGE (data not shown). Its relative activity was approximately 16000 APU/mg corresponded to that of protease BYA from *Bacillus* sp. Y. By contrast, no putative protease BYA was detected in the culture medium of transformants carrying pTA52B or pTA52C.

Site-Directed Mutagenesis and Enzymatic Analysis
Val29, Leu29, and Ile29 mutant protease BYA enzymes were generated by site-directed mutagenesis with primers (i), (j), and (k), respectively. Protease BYA (Ala29 enzyme) and its mutants were produced by pTA71 or by substitution at the Ala29 site, a transformant carrying pTA71 encoding Val29 mutant gene secreted 7200 APU/ml of alkaline protease in the culture medium shown in Table 2. The protease activity of culture medium increased approximately 1.4 fold greater than that of Ala29 enzyme. Each enzyme was purified by the method described by Shimogaki *et al.*,⁽⁶⁾ and its activity against casein and keratin was measured at the same enzyme concentrations, and their specific activity was measured in comparison with the Ala29 enzyme. As shown in Fig. 5, the results revealed approximately 150% greater specific activity of the Val29 mutant for each of the substrates as Ala29 enzyme, and that the Leu29 mutant and Ile29 mutant had activity nearly equal to Ala29 enzyme. Testing of the alkali resistance of each enzyme revealed residual activity of the Ala29 enzyme of approximately 900 APU/ml (90%), approximately 80% for the Val29 mutant, but only approximately 40% or

Fig. 6. Alkaline Stability of Protease BYA (Ala29) and Its Mutants (Val29, Leu29, Ile29)

Each protease (1000 APU/ml) was incubated in 0.1N NaOH at 35 °C for 30 min, residual activity was measured by the described method.⁽⁶⁾

Fig. 7. Stability of Protease BYA (Ala29) and Its Mutants (Val29, Leu29, Ile29) in Surfactant Solutions

Proteases (1000 APU/ml) were incubated at 35 °C for 20 h in 50 mM borate buffer (pH 10.5) containing each surfactant, and residual activity was measured by the described method.⁽⁶⁾ Gray bars indicate residual activity in sodium dodecyl sulfate (SDS), and solid bars indicate residual activity in sodium linear alkylbenzene sulfonate (LAS).

Fig. 8. Thermo-Stability of Protease BYA (Ala29) and Its Mutants (Val29, Leu29, Ile29)

Proteases (1000 APU/ml) were incubated at each temperature for 10 min in 50 mM borate buffer (pH 10.5). Residual activity was measured by the described method.⁽⁶⁾

less for the Leu29 mutant and Ile29 mutant (Fig. 6). The surfactant resistance of each enzyme was evaluated by comparing its residual activity after incubation in each surfactant solution for 20 h at 35 °C with its initial activity. The residual activity of each enzyme in sodium dodecyl sulfate (SDS) was 80% or more, but the residual activity of the Ala29 enzyme and Val29 mutant in linear alkyl benzene sulfate (LAS) was approximately 80%, as opposed to 20% or less for the Leu29 mutant and Ile29 mutant (Fig. 7). The heat resistance of each enzyme was evaluated by calculating its residual activity after incubation at different temperatures for 10 min as a per-

centage of its residual activity at 35 °C. The residual activity of all of enzymes was lower at 60 °C and above. The residual activity of the Ala29 enzyme was approximately 80%, as opposed to approximately 60% for the Val29 mutant, and the activity of the Leu29 mutant and the Ile29 mutant was almost completely lost at 65 °C (Fig. 8).

DISCUSSION

Amino Acid Sequence of Protease BYA The amino acid sequence of protease BYA was compared with that of several other subtilisins in Fig. 9. The amino acid sequence of protease BYA is approximately 160 residues longer than that of typical subtilisins, which consist of approximately 270 amino acid residues and have molecular masses of approximately 27000 Da, and most of the extra amino acids are

concentrated at the C-terminal and in several short peptides inserted near the active site. It can be thought that the presence of the inserted sequence and the large C-terminal peptide shown in Fig. 9 may be responsible for its alkaliphilic nature, its surfactants-resistance, and heat stability, although there is no conclusive evidence.

We registered the nucleotide sequence of protease BYA with Gene-Bank (No. E03808; Sep. 29, 1997). Saeki *et al.* discovered a protease, protease E-1, and registered its nucleotide sequence with Gene-Bank (No. AB046402; June 16, 2001).¹⁹⁾ As shown in Fig. 9, the amino acid sequence of protease E-1 is very similar to that of protease BYA. The only difference between them is 5 residues (amino acids no. +25, +63, +317, +328, +412). They also determined the DNA sequence of mature protease BYA and registered it with Gene-Bank (AB046404; Jan. 23, 2001) and they published

Fig. 9. Primary Structural Alignment of Protease BYA, Protease E-1,¹⁹⁾ Subtilisin E,²⁰⁾ Subtilisin Carlsberg,²⁴⁾ and H-221²⁵⁾

Each amino acid sequence is numbered starting with the N-terminal residue of the mature protein. Amino acids are represented by their single-letter code. Asterisks indicate amino acids that were identified as present in all of the sequences. Arrows point to the catalytic triad.

amino acid sequences.¹⁹⁾ The DNA sequence of the mature protease BYA reported by Saeki *et al.* also differs from ours by 4 nucleotides (nucleotides no. 896 to G, 1260 to A, 1724 to A, 1756 to A).

Expression of Protease BYA *E. coli* transformed with recombinant plasmid encoding the whole protease BYA gene expressed approximately 10 APU/ml of protease BYA into the culture medium. The amount produced was very low, about 1/30 of the amount produced by *Bacillus* sp. Y (data not shown). We therefore attempted to express protease BYA in *Bacillus subtilis* 1012 as the host, one of the mutant strains of *Bacillus subtilis* Marburg 168. It produced a little amount of subtilisin E, and we detected 320 APU/ml of subtilisin E in culture medium of its transformant with pUB110. First, expression was attempted by connecting the approximately 4.8 kbp DNA fragment encoding the protease BYA gene to pBR328 and pUB110 (pUY31). pUY31 is capable of replication in *E. coli* and genus *Bacillus*, but hardly any expression of protease BYA was observed. Since *Bacillus* sp. Y is an alkalophile, its regulatory mechanism of expression may be quite different from that of neutrophilic species of *Bacillus*. Since the homology between the deduced nucleotide sequence of the promoter region of protease BYA (1—216 bp) and subtilisin E²⁰⁾ is less than 50%, recognition of the protease BYA gene promoter in the host, *Bacillus subtilis* may be concluded to be low. There is a possibility of the higher expression by exchanging promoter that could be recognized well in the host. We then searched for a promoter that would be capable of sufficient expression in *Bacillus subtilis* and would be easy to connect to the structural gene of protease BYA. Both protease BYA and the amylase gene from *Bacillus licheniformis* have sequences that can be easily created *Bcl* I site adjacent their initial codons respectively. Moreover, the amylase can be produced in large amounts and has been produced as a commercial enzyme.²⁾ The *Bcl* I site was therefore constructed in each DNA fragment by site-directed mutagenesis, and after connecting amylase promoter and the structural gene, recombinant plasmids were constructed in order to express protease BYA. The transformant carrying pTA52A secreted 3400 APU/ml of alkaline protease. These results indicated that sufficient protease BYA can be expressed in *Bacillus subtilis* by replacing the promoter in common industrial culture medium. By contrast, hardly any expression of protease BYA was observed by transformants carrying pTA52B or pTA52C. As shown in Fig. 4, the distance between the SD and initial codon of pTA52A is the closest to that of the original amylase, and thus it appears necessary to make the SD-ATG initial codon distance as close as possible to the original distance in the amylase gene to achieve sufficient expression in *Bacillus subtilis* using the amylase promoter. Expression of protease BYA with pTA71 (7.63 Kb) was higher than with pTA52A (11.8 Kb). It can be indicated that the stability of the plasmid in a transformant was enhanced by miniaturization.

Site-Directed Mutagenesis of Protease BYA A number of studies on mutant subtilisins have been conducted by protein engineering.²¹⁾ Their 3-dimensional structure has been studied, and numerous results have been reported. Takagi *et al.*²²⁾ focused on amino acid Ile31, adjacent to Asp32, which is one of the amino acids that form the active site of subtilisin E, and they increased its specific activity 2—6 fold by

replacing its amino acid residues. Their findings suggested that amino acids in the vicinity of the active site are related in the reaction rate. Although protease BYA has been classified as a member of subtilisins, the same as subtilisin E, the alkali, heat, and surfactant resistance of protease BYA appear to be greater than that of subtilisin E. Amino acid sequence of protease BYA was different from that of common subtilisin, such as subtilisin E shown in Fig. 9. However, the vicinity of the active site of protease BYA is highly homologous with that of subtilisin E. Based on this finding, by replacing the amino acid residues in the vicinity of the active site, it appeared possible to make the enzyme reactivity similar to that of subtilisin E and retain these characteristic resistance properties of protease BYA. We therefore focused on the Ala29 located next to Asp30, which has been deduced to constitute one of the active site of protease BYA, and compared changes in its specific activity when its amino acid residues were replaced. Since Ala is a hydrophobic amino acid, we replaced it with Val, Leu, and Ile, which are classified as hydrophobic amino acids. The results showed that the specific activity of the Val29 mutant for casein and keratin as substrates was 1.5 fold higher than that of Ala29 enzyme. It can be thought that the property indicates improvement in detergency of soils containing keratin such as stain of underwear. Since the specific activity of the Leu29 mutant and the Ile29 mutant hardly differs at all from that of the wild type, the Val29 mutant alone may enhance the reaction rate. In addition, each of the enzymes was also evaluated on the heat, surfactants, and alkali resistance that are characteristic of protease BYA. Val29 mutant showed almost the same resistance to that of Ala29, and Leu29 mutant and Ile29 mutant showed lower resistance to that of Ala29. Although the mechanism is unknown, it can be thought that the side chain of amino acid 29 forms hydrophobic core with other hydrophobic side chains in vicinity of active site, and the conformation of the hydrophobic core affects specific activity and resistances.

CONCLUSION

It has been demonstrated that it is possible to express protease BYA in *Bacillus subtilis*. Studying replacements in promoters with high expression efficiency, increases in vector copy number and stability, and changes in host proliferation and culture conditions may make it possible to increase productivity even more in the future.

In this study it was possible to increase the specific activity of protease BYA by protein engineering without losing hardly any of the resistance properties that are its important characteristics. In the future it may be possible to produce even better enzymes for detergents by further improving substrate specificity and resistance ability by protein engineering. *Bacillus* sp. Y also produces a small amount of another alkaline protease in culture medium.²³⁾ It can be thought that its alkaline protease also be studied as well as protease BYA.

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