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Analysis of the Critical Sites for Protein Thermostabilization by Proline Substitution in Oligo-1,6-Glucosidase from *Bacillus coagulans* ATCC 7050 and the Evolutionary Consideration of Proline Residues

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To identify the critical sites for protein thermostabilization by proline substitution, the gene for oligo-1,6-glucosidase from a thermophilic *Bacillus coagulans* strain, ATCC 7050, was cloned as a 2.4-kb DNA fragment and sequenced. In spite of a big difference in their thermostabilities, *B. coagulans* oligo-1,6-glucosidase had a large number of points in its primary structure identical to respective points in the same enzymes from a mesophilic *Bacillus cereus* strain, ATCC 7064 (57%), and an obligately thermophilic *Bacillus thermoglucosidasius* strain, KP1006 (59%). The number of prolines (19 for *B. cereus* oligo-1,6-glucosidase, 24 for *B. coagulans* enzyme, and 32 for *B. thermoglucosidasius* enzyme) was observed to increase with the rise in thermostabilities of the oligo-1,6-glucosidases. Classification of proline residues in light of the amino acid sequence alignment and the protein structure revealed by X-ray crystallographic analysis also supported this tendency. Judging from proline residues occurring in *B. coagulans* oligo-1,6-glucosidase and the structural requirement for proline substitution (second site of the β turn and first turn of the α helix) (K. Watanabe, T. Masuda, H. Ohashi, H. Mihara, and Y. Suzuki, Eur. J. Biochem. 226:277–283, 1994), the critical sites for thermostabilization were found to be Lys-121, Glu-290, Lys-457, and Glu-487 in *B. cereus* oligo-1,6-glucosidase. With regard to protein evolution, the oligo-1,6-glucosidases very likely follow the neutral theory. The adaptive mutations of the oligo-1,6-glucosidases that appear to increase thermostability are consistent with the substitution of proline residues for neutrally occurring residues. It is concluded that proline substitution is an important factor for the selection of thermostability in oligo-1,6-glucosidases.

The proline rule for thermostabilizing proteins was proposed by Suzuki (22). This rule demonstrated that an increase in the frequency of proline occurrences at β turns and in the total number of hydrophobic residues can enhance protein thermostability. This idea was based on the finding that there is a strong correlation between an increase in the number of proline residues and the rise in the thermostability of oligo-1,6-glucosidases from *Bacillus* strains with different growth temperatures (*Bacillus cereus* ATCC 7064, 10 to 40°C; *Bacillus coagulans* ATCC 7050, 30 to 55°C; *Bacillus thermoamyloliquefaciens* KP1071, 30 to 66°C; *Bacillus thermoglucosidasius* KP1006, 42 to 69°C; and *Bacillus flavocaldarius* KP1228, 51 to 82°C) (23–26). Through further studies with oligo-1,6-glucosidase genes from *B. cereus* ATCC 7064 and *B. thermoglucosidasius* KP1006 (29, 30, 32) and through X-ray crystallographic analysis of *B. cereus* ATCC 7064 oligo-1,6-glucosidase (10, 31), it was determined that proline residues at the second sites of β turns and in the first turn of α helices also contributed to protein thermostability. Those proline residues mostly replaced hydrophilic or charged residues such as Glu and Lys. However, the original idea defining the proline rule was found to be conserved in these oligo-1,6-glucosidases. In the previous report, multiple proline substitutions introduced by site-directed mutagenesis on the oligo-1,6-glucosidase gene from *B. cereus* ATCC 7064 resulted in a cumulative thermostabilization of its protein product (33).

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We report here the cloning of the oligo-1,6-glucosidase gene from *B. coagulans* ATCC 7050, which possesses an intermediate thermostability between those of *B. cereus* ATCC 7064 and *B. thermoglucosidasius* KP1006. Through a structural comparison of these three oligo-1,6-glucosidases, we identified the proline residues that specifically occur in the *B. coagulans* ATCC 7050 oligo-1,6-glucosidase and also were able to point out four candidate residues in the *B. cereus* ATCC 7064 oligo-1,6-glucosidase that are believed to be important for thermostability in agreement with the proline rule. The data reported here further support the proline rule for thermostabilization of the oligo-1,6-glucosidase protein. Furthermore, we discuss protein evolution during oligo-1,6-glucosidase thermostabilization with reference to the neutral theory (8).

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *B. coagulans* ATCC 7050 was used as a source of chromosomal DNA. The *Escherichia coli* strains C600 (F^- *thi-1 thr-1 leuB6 lacY1 tonA21 supE44* λ^-) (19) and MV1184 {[*ara* Δ(*lac-proAB*) *rpsL thi* φ80 *lacZ* ΔM15] Δ(*srl-recA*) *306*::Tn*10* (Tet^r)/F′ (*traD36 proAB lacI*^{q} *lacZ*ΔM15)} (28) were used for screening and expression and for sequencing, respectively. Vector plasmids pUC18, for cloning, and pUC118/119, for sequencing with the helper phage M13 KO7, were used as previously described (34). L broth for *E. coli* cultivation consisted of 1% (wt/vol) peptone, 0.5% yeast extract, 0.5% NaCl, and 0.1% glucose in distilled water (pH 7.2). The medium for *B. coagulans* cultivation contained 2% soluble starch, 2% peptone, 0.2% yeast extract, 0.05% meat extract, 0.3% K_2HPO_4, and 0.1% KH_2PO_4 in distilled water (pH 7.0). Liquid cultivation was carried out aerobically as described previously (25).

Chromosomal DNA extraction and DNA manipulation. Chromosomal DNA was purified from *B. coagulans* ATCC 7050 cells grown at 50°C for 8 h on the medium described above, according to the method of Meade et al. (15). All DNA manipulations were performed as described by Sambrook et al. (19). All restriction and other modifying enzymes were used according to the suppliers' specifications.

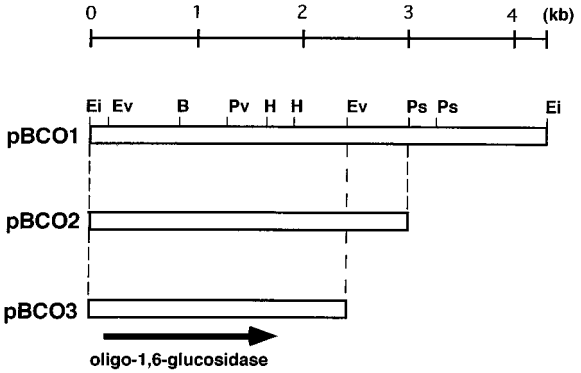

FIG. 1. Physical maps of the DNA fragments of the plasmids encoding the *B. coagulans* oligo-1,6-glucosidase gene. Open bars indicate the insert DNA in the hybrid plasmid carrying the oligo-1,6-glucosidase in pUC18. The location of the structural gene is designated by a thick arrow at the bottom. Restriction sites: B, *Bam*HI; Ei, *Eco*RI; Ev, *Eco*RV; H, *Hin*dIII; Pv, *Pvu*II; Ps, *Pst*I.

Selection for *E. coli* colonies containing the *B. coagulans* oligo-1,6-glucosidase gene. Chromosomal DNA (3.0 μg) of *B. coagulans* ATCC 7050 was partially digested with *Eco*RI and ligated into pUC18 (1.0 μg). After transformation of *E. coli* C600 cells with the ligation mixture, about 2,000 ampicillin-resistant colonies were obtained. All *E. coli* cells with or without pUC18, pUC118, and pUC119 showed neither an isomaltose-hydrolyzing *p*-nitrophenyl-α-D-glucopyranosidase active at 50°C nor a protein cross-reactive with rabbit antiserum against *B. coagulans* oligo-1,6-glucosidase on double immunodiffusion (30). A screening for *E. coli* C600 cells harboring the *B. coagulans* oligo-1,6-glucosidase gene was carried out by searching for *p*-nitrophenyl-α-D-glucopyranosidase activity of transformant colonies on blotting filter paper (16). The method was performed according to the procedure described in our previous papers (30, 32), except that the incubation temperature of the paper disk was 50°C. As a result of the screening for oligo-1,6-glucosidase, one yellow colony, the color of which was caused by the reaction of oligo-1,6-glucosidase on the substrate, was selected. The screened clone carried a plasmid, pBCO1, containing a 4.3-kb DNA insert in pUC18. The physical map of the DNA insert, as revealed by restriction enzyme analysis, is depicted in Fig. 1. Further subcloning resulted in two plasmids, pBCO2 and pBCO3, with inserts with sizes of 3.0 and 2.4 kb, respectively (Fig. 1).

Assay for the enzymatic activity of oligo-1,6-glucosidase. Oligo-1,6-glucosidase activity was determined spectrophotometrically at 50°C by monitoring the increase in A_{400} of a reaction mixture (1.0 ml) containing 33.3 mM phosphate buffer (pH 6.8), 2 mM *p*-nitrophenyl-α-D-glucopyranoside, and enzyme (25). One unit of activity was defined as the amount of enzyme hydrolyzing 1 μmol of *p*-nitrophenyl-α-D-glucopyranoside per min.

Amino acid sequencing. An Applied Biosystems (Foster City, Calif.) 477A gas–liquid-phase protein sequencer was used for the determination of the amino-terminal sequence of the *B. coagulans* oligo-1,6-glucosidase. The purified sample (100 μg) that reacted with phenylthiohydantoin was separated and identified with an Applied Biosystems on-line PTH 120A analyzer with a phenylthiohydantoin-C_{18} high-performance liquid chromatography column.

Purification of cloned oligo-1,6-glucosidase. All steps were carried out at 4°C, and centrifugation was done at 12,000 × *g* unless otherwise stated. *E. coli* C600 cells bearing pBCO3 were cultivated overnight at 37°C in 5 ml of L broth supplemented with 50 μg of ampicillin per ml. The culture (1 ml) was transferred into fresh medium (200 ml) and cultivated for 12 h. *E. coli* cells (wet weight, 14.2 g) obtained from the culture (total of 2 liters) by centrifugation for 10 min were suspended in 50 ml of buffer A (50 mM potassium phosphate, 5 mM EDTA [pH 7.0]) and disrupted by sonication at 4°C for 10 min. The cell debris was removed by centrifugation for 20 min. The sediments were then sonicated again. The cell extract (128 ml) was treated at 60°C for 30 min and centrifuged for 20 min. The supernatant was applied to a DEAE-cellulose column (5.0 by 20 cm) equilibrated with buffer A. Elution was performed at a rate of 20 ml/h with a linear gradient of 0 to 0.7 M NaCl in buffer A (2,000 ml). The active fractions were combined (380 ml), concentrated by ultrafiltration through a Diaflo-Amicon (Danvers, Mass.) PM-10 membrane and dialyzed against 150 mM phosphate–5 mM EDTA (pH 7.0 [buffer B]). The dialysate (20 ml) was loaded onto a Sephadex G-100 column (3.0 by 95 cm) equilibrated with buffer B. The column was developed with buffer B at the rate of 15 ml/h. The active fractions were combined (370 ml) and concentrated by ultrafiltration. The concentrate (74 ml) was dialyzed against 5 mM phosphate (pH 7.9) and applied to a hydroxylapatite (Bio-Gel HTP; Bio-Rad Laboratories, Richmond, Calif.) column (2.0 by 40 cm) equilibrated with the same buffer. After being washed with 120 ml of the same buffer, the column was eluted with 800 ml of a linear 5 to 50 mM phosphate buffer gradient. The active fractions were pooled (370 ml), concentrated by ultrafiltration, and dialyzed against buffer A. The dialysate was subjected to gel filtration on a Bio-Gel P200 (Bio-Rad Laboratories) column (3.0 by 95 cm) equilibrated with buffer A. The column was developed with the buffer at a rate of 10 ml/h. The active fractions were combined (92.5 ml) and concentrated to 30 ml by ultrafiltration.

DNA sequencing. Progressive unidirectional deletions containing overlapping sequences of the inserted DNA fragment (2.4-kb *Eco*RI-*Eco*RV fragment) encoding the oligo-1,6-glucosidase gene were created with the *Exo*III/mung bean nuclease kit (Takara Shuzo, Kyoto, Japan). The single-stranded template DNA was obtained with a helper phage, M13 KO7, and was used for the DNA sequencing reaction (20).

Nucleotide sequence accession number. The nucleotide sequence reported here has been deposited in the GenBank, EMBL, and DDBJ databases under accession number D78342.

RESULTS

Cloning and sequencing of the gene encoding oligo-1,6-glucosidase. By following the procedure described in Materials and Methods, the gene responsible for oligo-1,6-glucosidase from *B. coagulans* ATCC 7050 was cloned as a 4.3-kb DNA insert in pUC18 (pBCO1 [Fig. 1]). The *E. coli* cells harboring pBCO1 clearly showed *p*-nitrophenyl-α-D-glucopyranosidase activity on the blotting paper.

The 2.4-kb *Eco*RI-*Eco*RV fragment from pBCO1 was inserted between the *Eco*RI and *Sma*I sites of pUC118 and pUC119 for DNA sequencing. With deletion clones generated from both plasmids, the DNA sequences of both strands were determined (Fig. 2). The *Eco*RI-*Eco*RV fragment was found to be 2,352 bp in length and contained a single open reading frame with a size of 1,668 bp. The open reading frame corresponded to a polypeptide with a size of 555 amino acids and with a predicted molecular mass of 64,953 Da. The sequence of the first 15 amino-terminal amino acids coincided with that of the cloned oligo-1,6-glucosidase purified from *E. coli* cells bearing pBCO3. The amino-terminal amino acid had been previously determined to be threonine for the native oligo-1,6-glucosidase purified from *B. coagulans* ATCC 7050 (25). Since threonine occurred as the second amino acid, the first amino acid, methionine, might have been processed at the amino terminus of oligo-1,6-glucosidase in *B. coagulans* ATCC 7050 cells. The start codon, ATG, was located at nucleotide positions 48 to 50, close to the *Eco*RI cloning site, and the stop codon, TAG, was located at nucleotide positions 1713 to 1715. A Shine-Dalgarno sequence was assigned as GGAGTAT GAG, 5 bp upstream of the start codon. No consensus promoter sequences could be found in the 5′-flanking region upstream of the open reading frame. It seems reasonable that in the *E. coli* host lacking *lacI*^{q}, the *lac* promoter of pUC18 must be responsible for the expression of the oligo-1,6-glucosidase. This promoter would have the correct spacing and is in the correct orientation for the expression of the cloned gene.

The putative amino acid composition of oligo-1,6-glucosidase from *B. coagulans* was compared with that of the sequence experimentally determined for the native oligo-1,6-glucosidase purified from *B. coagulans* ATCC 7050 (25). The putative amino acid compositions of *B. cereus* ATCC 7064 and *B. thermoglucosidasius* KP1006 oligo-1,6-glucosidases, deduced from their nucleotide sequences (29, 32), are also listed in Table 1. The putative oligo-1,6-glucosidase of *B. coagulans* ATCC 7050 was in good agreement with the experimental sequence of the native enzyme, except for the percentage of cysteine. Comparison of the amino acid composition of the *B. coagulans* oligo-1,6-glucosidase with those of the *B. cereus* and *B. thermoglucosidasius* oligo-1,6-glucosidases showed that the enzymes were highly similar, with the exception of the remarkable increase in proline, the occurrence of which coincided with an increase in the thermostability of the corresponding proteins.

1
GAATTCATCCATCTAGCAAATACGTACACGAAGGAGTATGAGCCTTC
65 100 SD
ATG ACA GAA TGG TGG AAA AAA GCC GTT GTT TAC CAG ATT TAC CCG CGC AGT TTT TAT GAT ACA AAT GGT GAC GGG
M T E W W K K A V V Y Q I Y P R S F Y D T N G D G 25
140 175
ATC GGC GAT TTG CGG GGA ATT ATG GAT AAG CTC GAC TAT TTG AAA ACA CTC GGG ATC GAC TGC ATC TGG ATC AGC
I G D L R G I M D K L D Y L K T L G I D C I W I S 50
215 250
CCG GTG TAC GAC TCG CCG CAG GAT GAC AAT GGC TAC GAT ATC CGT GAC TAC CGC AAG ATC GAC AAG ATG TTC GGG
P V Y D S P Q D D N G Y D I R D Y R K I D K M F G 75
290 325
ACA AAT GAA GAT ATG GAC CGC CTG CTT GAC GAA GCC CAT GCA CGC GGG ATC AAA ATC GTA ATG GAT CTT GTC GTG
T N E D M D R L L D E A H A R G I K I V M D L V V 100
365 400
AAC CAT ACC TCC GAT GAA CAT GCC TGG TTT GTC GAA AGC CGG AAA TCG AAG GAT AAT CCG TAC CGC GAC TTT TAT
N H T S D E H A W F V E S R K S K D N P Y R D F Y 125
440 475
TTT TGG AAA GAC CCG AAA CCG GAC GGC ACC CCT CCG AAC AAC TGG GGT TCG ATG TTT TCC GGT TCC GCC TGG GAG
F W K D P K P D G T P P N N W G S M F S G S A W E 150
515 550
TAC GAC GAA ACA ACC GGG CAA TAC TAT TTG CAC TAT TTT TCA AAG AAA CAG CCT GAC TTA AAC TGG GAA AAC GAA
Y D E T T G Q Y Y L H Y F S K K Q P D L N W E N E 175
590 625
AAA GTG CGG AAA GAG ATT TAC GAT ATG ATG AAG TTC TGG ATG GAT AAA GGG GTG GAC GGC TGG CGC ATG GAT GTG
K V R K E I Y D M M K F W M D K G V D G W R M D V 200
665 700
ATC GGC TCG ATT TCC AAG TTT CTT GAT TTT CCT GAT TAT GAA CTT CCG GAA GGG CAA AAA TAC GGC ATT GGC AAG
I G S I S K F L D F P D Y E L P E G Q K Y G I G K 225
740 775
TAT CAT GCA AAC GGG CCG CGC CTC CAT GCG TTC ATC CAG GAA ATG AAC CGC GAA GTG CTG TCA AAA TAC GAC TGC
Y H A N G P R L H A F I Q E M N R E V L S K Y D C 250
815 850
ATG ACT GTC GGG GAA GCC ATC GGA TCC GAT GTC GAA ATC GCC AGG AAA TAT ACA GGG CCG GAC CGC CAT GAA CTC
M T V G E A I G S D V E I A R K Y T G P D R H E L 275
890 925
AAT ATG ATT TTT AAT TTT GAA CAT ATG GAT GTC GAT ACG AAA CCG GGC AGC CCT GCC GGC AAA TGG GCT TTG AAG
N M I F N F E H M D V D T K P G S P A G K W A L K 300
965 1000
CCT TTT GAC CTG GTC GAA TTG AAA CAA ATC CTT TCC CGC TGG CAA TAT GAG CTC GCG GAT ACC GGC TGG AAT GCG
P F D L V E L K Q I L S R W Q Y E L A D T G W N A 325
1040 1075
CTC TAT TTT GAA AAC CAT GAC CAG GCG AGG GTG GTA TCG CGC TGG GGC AAT GAT ACA ACG TAC CGG GCA GAA TGT
L Y F E N H D Q A R V V S R W G N D T T Y R A E C 350
1115 1150
GCC AAA GCG TTT GCC ACC ATT TTG CAC GGG CTG AAA GGG ACC CCT TTT ATT TAT CAA GGG GAA GAA ATC GGG ATG
A K A F A T I L H G L K G T P F I Y Q G E E I G M 375
1190 1225
GTG AAC GCC GAT CTC GAA CTC GAA GAA TAT GAT GAC ATT GAA ATC CGG AAT GCT TAT CAA GAG CTT GTG ATG GAA
V N A D L E L E E Y D D I E I R N A Y Q E L V M E 400
1265 1300
AAC CAA ATC ATG TCG AAA GAT GAA TTT TTA ACA GCT GTC CGA AAA AAA GGA CGC GAC AAT GCA CGG ACG CCG ATG
N Q I M S K D E F L T A V R K K G R D N A R T P M 425
1340 1375
CAG TGG GAC GGC AGT TTT AAT GCC GGC TTT ACA ACC GGA ACA CCG TGG CTT AAA GTC AAT TCC CGC TAT TCG GAG
Q W D G S L N A G F T T G T P W L K V N S R Y S E 450
1415 1450
ATC AAC GTG GCA AAA GCG CTT CAA GAG CCT GAT TCG ATT TTT TAT TAC TAT CAA TCT TTG ATT AAG CTG CGG CAT
I N V A K A L Q E P D S I F Y Y Y Q S L I K L R H 475
1490 1525
TCA TAC GAT GTG TTT ACA GAC GGC CGG TAT GAG CTG CTG ATG CCG GAC CAT CCG CAT TTG TAC GTG TAC ACG AGG
S Y D V F T D G R Y E L L M P D H P H L Y V Y T R 500
1565 1600
GAA AAT GAA TCG GAA AAA CTG CTC GTT GCC GCC AAC TTA AGC GAA AAC ACA GTA AGC TTT GAT CAA CCG GAT GAC
E N E S E K L L V A A N L S E N T V S F D Q P D D 525
1640 1675
AAC TGG AAA CTG CTG CTT GGA AAC TAT GAA GAT ACT GGC ACA AGC ACG CTG TTC CGC CCG TAT GAA GCG GCC ATT
N W K L L L G N Y E D T G T S T L F R P Y E A A I 550
1715 1750
TAC TAT CTG GAA AAA TAG TTTTTTGCGTGCGGTTTTTGCCCGATGGCCTTTTTATGCAAAGAGCCTCCATACTACGTATGAGGGGCTCTTTGCT
Y Y L E K ***
1800 1850
GTTTCCTTTATGAAACTGTAATGGCCTTTACCACTTCTTCTAGCTGCATGCCTCTTGAGGCTTTGACCAGAATCAAATCGCCGGCTCCGATTTTTCCTTT
1900 1950
CAACATATCGATCAACCGCTCCTTGTCCTCAAACGCAAACACGCGGTTGCCCGGAAAATCGCAAGCTTCCGCGATCAAACGGCCCAACTTGCCATATGTA
2000 2050
AAAACAAAATCAATTTTTTCAGGGTTCAAGCTGCGGCCGACCTCCCTGTGAAACGCCGCTTCCTTTTCACCTAGTTCAAGCATATCCCCGAGCACGAGGA
2100 2150
TTTTCCGGCCCCGTTCCTTCAAACTGCTCCACAAGCCGGATGGCCGCACGCATCGACGTCGGGCTTGCATTATATGCATCGTTGATGATTTTTGAACCGTT
2200 2250
GATGCCATCCGCCATTTCCATCCGCATTGCGCTCAGCCTCGTTTTCCCTAGGCCTGCGCGGATTTCCTCAAATGCATGCCAAACTCGCGGCCGGCAAGAA
2300 2352
TGGCGGCAAGCGAATTGATAACATTGTATTCGCCAAGGACAGGCAGATGAAAACTGATATC

FIG. 2. DNA and computer-generated amino acid sequence of the 2.4-kb DNA insert harboring the *B. coagulans* oligo-1,6-glucosidase gene. Nucleotides of the 2.4-kb *Eco*RI-*Eco*RV DNA fragment are shown with consecutive numbering starting from the 5′ terminus. The putative Shine-Dalgarno (SD) ribosome binding sequence is underlined.

Structural homology with other oligo-1,6-glucosidases. The primary structure deduced from the *B. coagulans* oligo-1,6-glucosidase gene was compared with those of the other two oligo-1,6-glucosidases (Fig. 3). The percentages of identity of *B. coagulans* oligo-1,6-glucosidase to the *B. cereus* and *B. thermoglucosidasius* oligo-1,6-glucosidases were 57 and 59%, respectively. These values were slightly lower than that from the comparison between the *B. cereus* and *B. thermoglucosidasius* oligo-1,6-glucosidases (72%) (29).

On the basis of the amino acid sequence alignment of these three oligo-1,6-glucosidases and the results of the X-ray crystallographic analysis of *B. cereus* oligo-1,6-glucosidase (10), the secondary structural elements of the *B. coagulans* oligo-1,6-glucosidase were assigned as shown in Fig. 3 and Table 2. Neither additional nor missing secondary structure was observed in the *B. coagulans* oligo-1,6-glucosidase. Therefore, these secondary structural elements should permit the *B. coagulans* oligo-1,6-glucosidase to assume a tertiary structure, analogous to that of the other two oligo-1,6-glucosidases, with respect to an N-terminal domain [$(\beta/\alpha)_8$ motif (1)], a subdomain, and a C-terminal domain (10).

The catalytic residues of the *B. cereus* and *B. thermoglucosidasius* oligo-1,6-glucosidases were proposed to be Asp-199, Glu-255, and Asp-329 for the *B. cereus* oligo-1,6-glucosidase and Asp-199, Glu-256, and Glu-330 for the *B. thermoglucosidasius* oligo-1,6-glucosidase (29, 32). X-ray crystallographic analysis revealed that these active residues are located in the cleft between the N-terminal domain and the subdomain (10). With site-directed mutagenesis to alter these residues of the *B. cereus* oligo-1,6-glucosidase gene, we could show through the loss of activity that these three residues were critical to the catalytic behavior of this protein. These equivalent residues (Asp-199, Glu-255, and Asp-332) were completely conserved in the *B. coagulans* oligo-1,6-glucosidase. Not only are the three catalytically active site residues conserved, but so are a number of other residues in the vicinity of the catalytic site (29). These observations support the idea that all of the oligo-1,6-glucosidases share the same catalytic mechanism.

TABLE 1. Comparison of amino acid compositions of *B. coagulans*, *B. cereus*, and *B. thermoglucosidasius* oligo-1,6-glucosidases

Amino acid	Amino acid composition [no. of residues (mol %)] of oligo-1,6-glucosidase from:			
	B. coagulans[a]		*B. cereus* (putative)[b]	*B. thermoglucosidasius* (putative)[b]
	Putative	Experimental		
Ile	29 (5.23)	28 (5.38)	37 (6.63)	34 (6.05)
Phe	23 (4.14)	22 (4.30)	19 (3.41)	17 (3.02)
Pro	24 (4.32)	24 (4.63)	19 (3.41)	32 (5.69)
Leu	42 (7.57)	38 (7.23)	39 (7.00)	38 (6.76)
Val	26 (4.68)	23 (4.36)	32 (5.73)	34 (6.05)
Met	19 (3.42)	16 (3.09)	28 (5.02)	24 (4.27)
Ala	29 (5.22)	26 (5.05)	14 (2.51)	16 (2.85)
Gly	37 (6.67)	36 (6.93)	36 (6.45)	37 (6.58)
Cys	3 (0.54)	0 (0)	3 (0.54)	0 (0)
Tyr	37 (6.67)	31 (6.02)	35 (6.27)	39 (6.94)
Lys	37 (6.67)	35 (6.74)	41 (7.35)	42 (7.47)
Arg	27 (4.86)	24 (4.63)	20 (3.58)	33 (5.87)
Thr	27 (4.86)	24 (4.63)	23 (4.12)	23 (4.09)
Ser	28 (2.34)	25 (4.76)	28 (5.02)	28 (4.98)
His	13 (2.34)	19 (3.57)	16 (3.16)	11 (1.96)
Asn	28 (2.34)		38 (6.81)	28 (4.98)
Asp	52 (9.37)		37 (6.63)	45 (8.01)
Asx	80 (14.4)	77 (14.8)	75 (13.4)	73 (13.0)
Gln	16 (2.88)		15 (2.69)	14 (2.49)
Glu	41 (7.39)		62 (13.8)	50 (8.90)
Glx	57 (10.3)	60 (11.5)	77 (16.5)	64 (11.4)
Trp	17 (3.06)	12 (2.31)	16 (2.87)	17 (3.02)

[a] The putative and experimental values for *B. coagulans* oligo-1,6-glucosidase were derived from this study and the previous paper (25), respectively.

[b] The putative values for *B. cereus* and *B. thermoglucosidasius* oligo-1,6-glucosidases are quoted from previous papers (29, 32), respectively.

Purification and characterization of the cloned *B. coagulans* oligo-1,6-glucosidase. In order to study the products of the cloned and native oligo-1,6-glucosidases, the cloned oligo-1,6-glucosidase was expressed in *E. coli* C600 cells, purified, and characterized. A six-step procedure was performed for the purification, as shown in Table 3. The final preparation (23.2 ml [119 mg]) contained 345 U/mg of protein, which was 12.7-fold enhanced over the concentration of the cell extract; the yield was 48%. The cloned oligo-1,6-glucosidase exhibited a mobility identical to that of the native oligo-1,6-glucosidase according to both native and sodium dodecyl sulfate-denatured polyacrylamide gel electrophoresis (data not shown). The isoelectric point for the cloned oligo-1,6-glucosidases was 4.1, which was close to that of the native enzyme (4.3) in the previous paper (25). Double immunodiffusion of the cloned oligo-1,6-glucosidase was performed with rabbit antiserum raised against the native oligo-1,6-glucosidase (25) and resulted in the formation of the precipitin lines without any spur (data not shown).

The temperature dependence of the native and cloned oligo-1,6-glucosidases on activity was examined (Fig. 4A). The activities of both oligo-1,6-glucosidases were maximal at 62°C, with half-optima at 48 and 67°C. Neither protein showed activity at 78°C. The thermostabilities of both oligo-1,6-glucosidases were compared by monitoring the remaining activity after incubation in buffer A for 10 min at various temperatures (Fig. 4B). With these data, the temperature at which 50% activity was lost (T_m) was determined as 61.0°C for both oligo-1,6-glucosidases. As a result, the profiles of the T_ms of the various oligo-1,6-glucosidase-versus-proline contents were obtained and are depicted in Fig. 4C.

The inhibitory effects by heavy metal ions were investigated with the glucosidase assay described in Materials and Methods. Reaction mixtures contained either 2 mM heavy metal ion (added as the salt of chloride) or 2 mM EDTA in 20 mM H_3BO_3-KCl-NaOH buffer (pH 7.5). Both native and cloned oligo-1,6-glucosidase demonstrated similar inhibitory effects with respect to the various heavy metal ions and other reagents tested. These values included the following: Cd^{2+}, 100%; Pb^{2+}, 100%; Co^{2+}, 95%; Hg^{2+}, 100%; Cu^{2+}, 100%; Ni^{2+}, 81%; Fe^{2+}, 99%; Ba^{2+}, 28%; Mg^{2+}, 25%; Ca^{2+}, 25%; Sr^{2+}, 11%; and EDTA, 0%. The thiol reagents *p*-chloromercuribenzoic acid and 5,5′-dithiobis(2-nitrobenzoic acid) had inhibitory effects of 35 and 3%, respectively, after preincubation at 53.3 μM with the enzyme for 20 min at 50°C (25). Because the *B. coagulans* oligo-1,6-glucosidase contains three cysteine residues, the inhibition by thiol was reasonable. The cloned oligo-1,6-glucosidase showed the K_m and k_0 values for *p*-nitrophenyl-α-D-glucopyranoside (0.17 mM, 253 s^{-1}) and isomaltose (2.1

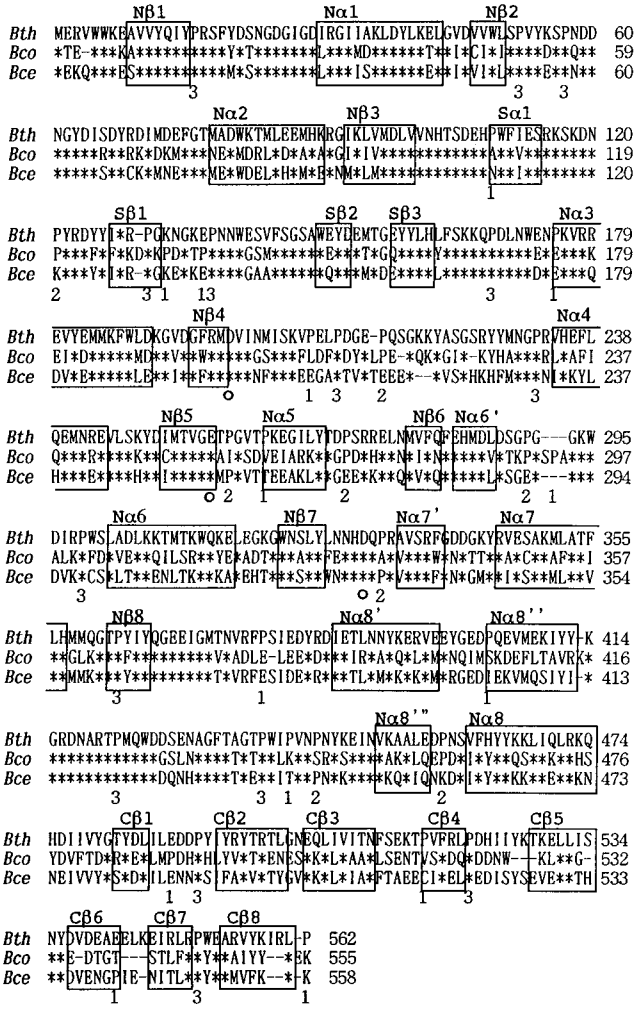

FIG. 3. Comparison of the primary sequences and secondary structural assignments of three bacillary oligo-1,6-glucosidases. The sequences are shown in the order *B. thermoglucosidasius* oligo-1,6-glucosidase (*Bth*), *B. coagulans* oligo-1,6-glucosidase (*Bco*), and *B. cereus* oligo-1,6-glucosidase (*Bce*). Identical residues of the three oligo-1,6-glucosidases are symbolized by asterisks in the second and third rows. Bars represent gaps introduced during the alignment process. Each secondary structural element is boxed with the names used in previous reports (10, 33). The number of proline occurrences is given under the primary sequences. Putative active site residues are marked with o.

TABLE 2. Positions of secondary structures and unique prolines

Secondary structure or unique proline	Position of secondary structure or proline for oligo-1,6-glucosidase from:		
	B. cereus ATCC 7064	*B. coagulans* ATCC 7050	*B. thermoglucosidasius* KP1006
Secondary structures[a]			
Nβ1	9–15	8–14	9–15
Nα1	30–43	29–42	30–42
Nβ2	47–50	46–49	47–50
Nα2	78–90	77–89	78–90
Nβ3	93–100	92–99	93–100
Nα3	175–190	175–190	175–190
Nβ4	195–198	195–198	195–198
Nα4	233–243	233–243	234–244
Nβ5	250–255	250–255	251–256
Nα5	261–267	261–267	262–268
Nβ6	277–280	277–280	278–281
Nα6′	282–286	282–286	283–287
Nα6	301–314	304–317	302–315
Nβ7	320–324	323–327	321–325
Nα7′	333–337	336–340	334–338
Nα7	344–356	347–359	345–357
Nβ8	361–365	364–368	362–366
Nα8′	386–397	388–399	387–398
Nα8″	403–412	405–414	404–413
Nα8‴	450–455	453–458	451–456
Nα8	460–473	463–476	461–474
Sα1	109–114	108–113	109–114
Sβ1	127–131	126–131	127–131
Sβ2	149–152	149–152	149–152
Sβ3	157–161	157–161	157–161
Cβ1	481–484	484–487	482–485
Cβ2	492–499	495–502	493–500
Cβ3	502–509	505–512	503–510
Cβ4	515–518	518–521	516–519
Cβ5	527–533	528–532	528–534
Cβ6	536–540	535–539	537–541
Cβ7	544–547	540–544	546–550
Cβ8	552–557	548–553	554–561
Unique proline	541	132, 136, 293, 490	109, 175, 208, 262, 379, 404, 441, 516, 562

[a] Secondary structure assignments were derived from Fig. 3.

mM, 229 s^{-1}), respectively. These kinetic parameters were in good agreement with those of the native oligo-1,6-glucosidase (25). These results indicated that the cloned oligo-1,6-glucosidase from *B. coagulans* ATCC 7050 was identical to the native enzyme in *E. coli* cells.

DISCUSSION

Since the proline theory based on comparative analysis of various bacillary oligo-1,6-glucosidases was demonstrated (22, 24), protein thermostabilization by a strategy of proline substitution was reported to be applicable for many proteins. It was first shown that proline substitution in bacteriophage T4 lysozyme enhanced its thermostability (14). Replacement of Ala-87 in T4 lysozyme with a proline residue at a β turn decreased the backbone entropy of unfolding. This entropic effect contributed to the increase in the free energy change for protein thermostabilization. Thereafter, similar results with respect to proline substitution at single sites were reported for hen egg lysozyme (27), human lysozyme (4), *E. coli* ribonuclease HI (7, 9), and *Bacillus stearothermophilus* neutral protease (3). Recently, cumulative enhancement of thermostability by multiple proline substitutions provided conclusive evidence

TABLE 3. Purification of oligo-1,6-glucosidase from *E. coli* C600 cells bearing pBCO3

Fraction	Total		Sp act (U/mg)
	Protein (mg)	Activity (U)	
Cell extract	3,140	85,500	27.2
Heat treatment	2,000	81,700	40.9
DEAE-cellulose eluate	688	79,800	116
Sephadex G-100 eluate	212	58,200	275
Hydroxylapatite eluate	150	50,500	336
Bio-Gel P-200 eluate	119	41,100	345

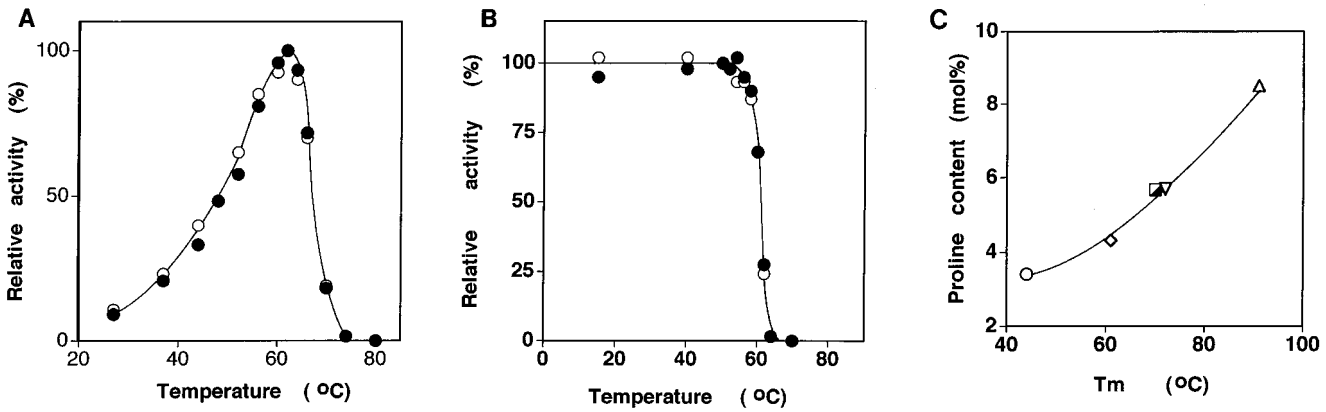

FIG. 4. (A) Effect of temperature on the activities of the cloned (○) and native (●) oligo-1,6-glucosidases from *B. coagulans* ATCC 7050. The enzyme activities were determined as described in Materials and Methods. The activity was expressed as the percentage of the maximum value at 62°C. (B) Effect of temperature on the stability of the cloned (○) and native (●) oligo-1,6-glucosidases from *B. coagulans* ATCC 7050. The enzyme (1.15 μg) was incubated for 10 min in 50 μl of buffer A, diluted with 0.15 ml of buffer A, and then assayed for its remaining activity. The activity observed at 50°C was defined as 100%. (C) Strong correlation between the increase in the proline contents of five *Bacillus* oligo-1,6-glucosidases and the increase in their thermostability (T_m [Tm]). Thermostability is expressed as the temperature at which the enzyme (15 to 23 μg/ml) was 50% inactivated in 10 min at pH 6.8. The proline contents of the oligo-1,6-glucosidases from *B. cereus* ATCC 7064 (○), *B. thermoamyloliquefaciens* KP1071 (◪), *B. thermoglucosidasius* KP1006 (▽), and *B. flavocaldarius* KP1228 (△) are quoted from previously published reports (24, 29, 32). The data for *B. coagulans* oligo-1,6-glucosidase (◇) are from this study.

that (i) proline residues critical for thermal stabilization favor second sites of β turns and the first turns of α helices, (ii) most critical sites occur randomly over the protein surface, and (iii) substituted prolines independently contribute to thermal stabilization (33). By following this strategy in combination with other evolutionary improvements in various factors, *Bacillus* strains growing in various ranges of temperature are supposed to accomplish the thermal adaptation of oligo-1,6-glucosidase in nature and give clues about the critical sites and structures for proline substitution in their oligo-1,6-glucosidases.

This study revealed that the *B. coagulans* ATCC 7050 oligo-1,6-glucosidase contained 24 proline residues. The proline content was comparable to that calculated from the amino acid composition of the native enzyme purified from *B. coagulans* ATCC 7050. The correlation of proline content with protein thermostability was shown to be consistent, as illustrated previously (Fig. 4C). The proline residues of the *B. cereus* and *B. thermoglucosidasius* oligo-1,6-glucosidases and those of the *B. coagulans* oligo-1,6-glucosidase were classified according to their primary sequence alignment (Fig. 3) and protein structure (Table 2). The results are depicted in Fig. 5. These three oligo-1,6-glucosidases share 15 common proline residues corresponding to the following residue numbers for the *B. cereus* oligo-1,6-glucosidase: 16, 52, 57, 130, 137, 168, 211, 231, 298, 362, 421, 437, 490, 520, and 549. Moreover, the three proline residues of the *B. cereus* oligo-1,6-glucosidase (257, 331, and 443) and five proline residues of the *B. coagulans* oligo-1,6-glucosidase (120, 216, 270, 290, and 460) were shared between the *B. cereus* and *B. thermoglucosidasius* oligo-1,6-glucosidases and between the *B. coagulans* and *B. thermoglucosidasius* oligo-1,6-glucosidases, respectively. However, none of these eight proline residues were observed to be shared between the *B. cereus* and *B. coagulans* oligo-1,6-glucosidases. There are also a number of prolines unique to each of the oligo-1,6-glucosidases. The thermostable *B. thermoglucosidasius* protein has nine unique proline residues at positions 109, 175, 208, 262, 379, 404, 441, 516, and 562. The *B. coagulans* protein, which is intermediate in thermostability, has four unique proline residues at positions 132, 136, 293, and 490. Interestingly, there is only one unique proline residue, at amino acid position 541, in the thermolabile oligo-1,6-glucosidase of *B. cereus* ATCC 7064. These observations revealed that the occurrence of proline consistently increased along with the rise in thermostability of oligo-1,6-glucosidases. Not including the 15 shared proline residues or Pro-293 in the *B. coagulans* oligo-1,6-glucosidase, we note that these extant proline residues are at sites predicted by the proline rule to be critical to the thermostabilization of the proteins.

Nine of these critical sites in the *B. cereus* oligo-1,6-glucosidase, which also occur in the *B. coagulans* enzyme, were structurally characterized by direct comparison with the structure of the *B. cereus* oligo-1,6-glucosidase that was determined by X-ray crystallographic analysis (10). Pro-132, Pro-136, Pro-216, and Pro-270 in the *B. coagulans* enzyme were equivalent to the residues Lys-132, Glu-136, Glu-216, and Glu-270 in *B. cereus*

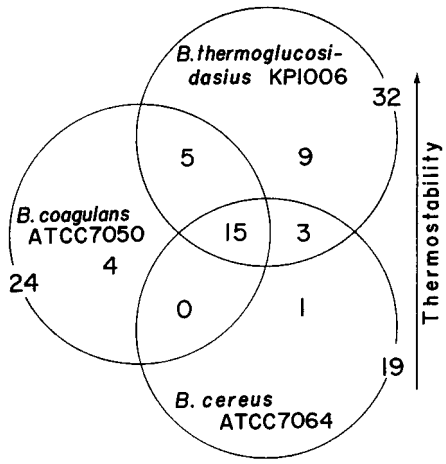

FIG. 5. Classification of common and uncommon proline residues of the three oligo-1,6-glucosidases compared in this study. The vertical direction shows the increase in thermostability in an arbitrary unit. The three circles represent the *B. thermoglucosidasius*, *B. coagulans*, and *B. cereus* oligo-1,6-glucosidases. The numbers shown within the regions created by these circles indicate the number of proline residues shared by these oligo-1,6-glucosidases. The numbers on the circles are the respective total numbers of proline residues in individual oligo-1,6-glucosidases.

oligo-1,6-glucosidase and located in the flexible loops. Pro-293, lying in a three-amino-acid insert between Nα6′ and Nα6 in *B. coagulans* oligo-1,6-glucosidase, was also presumably in a flexible loop according to the secondary structure prediction (2). The effect of proline substitution of these residues in flexible loops may be slighter than that of other residues, such as Glu-216 and Glu-270, for which the significance was unclear (33). The sites (Lys-121, Glu-290, Lys-457, and Glu-487) in the *B. cereus* oligo-1,6-glucosidase corresponding to Pro-120, Pro-290, Pro-460, and Pro-490 in the *B. coagulans* oligo-1,6-glucosidase were at the second sites of β turns. These four sites are good candidates for thermostabilization by proline substitution. In fact, proline substitution at Lys-121 and Glu-290 resulted in additive enhancement of its thermostability (33). However, the conformation of a residue preceding the substituted proline residue is also an important factor (5) for thermostabilization. Since Leu-486, preceding Glu-487, has an unfavorable conformation angle in the region of αL ($\phi = 44°$, $\psi = 45°$) (5, 21), proline substitution at 487 may be less effective for thermostabilization. In addition to the structural characteristics, the residues in the *B. cereus* oligo-1,6-glucosidase equivalent to specific prolines in the *B. coagulans* oligo-1,6-glucosidase are limited to all hydrophilic or ionic ones, such as Glu and Lys. This finding well follows the definition of the proline rule.

It is likely that the 15 proline residues shared among the three oligo-1,6-glucosidases are essential for maintaining the structure and function of the protein. Proline is such a peculiar amino acid, because it has an intramolecular pyrrolidine ring between the α-carbon and the preceding peptide bond nitrogen (12). Hence, the influence of proline on other residues in the vicinity is so strong that proline has a preference for the second sites of β turns, the first turns of α helices, and the flexible loops in proteins (12, 18). If such a proline residue is imprudently substituted for other amino acids, it may result in the destruction of both secondary and tertiary structures as well as the loss of protein function and thermostability (6). This is the case for the application of the proline rule at unsuitable sites, such as the middle of an α helix (11).

The neutral theory states that sequence changes in proteins are caused by fixation of selectively neutral mutations (8). Malcolm et al. (13) examined a selectively neutral corridor with an in vitro system by determining the T_ms for extant sequences of bird lysozymes. Since some of the mutant lysozyme proteins representing intermediates along the possible evolutionary pathways had thermostabilities outside the range of the extant proteins, Malcolm et al. concluded that the evolution of lysozyme follows a nonneutral evolutionary pathway (13). On the other hand, the findings of a similar study with *Saccharomyces cerevisiae* cytochrome *c* were consistent with the neutral theory of evolution (17). With respect to the oligo-1,6-glucosidase, we have shown previously (33) and again in this study (Fig. 4C) data which support an evolutionary pathway consistent with the neutral theory. Upon comparison of the T_ms from this study with those of extant sequences, five oligo-1,6-glucosidases from various *Bacillus* strains demonstrated thermostabilities within the corridor defined by most thermostable and thermolabile oligo-1,6-glucosidases. This is consistent with what we observed before—that the thermostabilities of mutant oligo-1,6-glucosidases containing substituted prolines lie significantly within the thermostability corridor. Therefore, the evolution of oligo-1,6-glucosidases supports the neutral theory of evolution for proline substitution. While our data are as yet quite limited, these findings suggest that proline substitution is an important factor in the selection of thermostability during the evolution of oligo-1,6-glucosidase.

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