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Structure of *Bacillus* α -glucosidase: nucleotide sequence of the gene and deletion analysis

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Primary structure of α –glucosidase (α -glucoside glucohydrolase: EC3.2.1.20) of *Bacillus stearothermophilus* MIB010 was deduced from its nucleotide sequences (1665 base pairs). The amino acid sequence of the enzyme consisted of 555 residues with a molecular weight of 65233. After truncating the C-terminus end the deleted and subcloned genes were subjected to the expression in the host cells. The deletion analysis indicated that the C-terminal regions were crucial for the enzymatic activity.

Keywords: glucosidase, *Bacillus stearothermophilus*, *p*-nitrophenyl- α-D-glucopyranoside (*p*NPG), gene cloning, immunochemical detection, Western blotting

Starch degradation product of great interest was maltose and glucose because of their mild sweetness, stability and little tendency to crystallize[1]. Several α-amylases have been employed to hydrolyze starch into a mixture of oligosaccharides which in turn must be further degraded to maltose and glucose. It was of interest to establish single enzyme system, which convert starch effectively to its constituents.

Bacillus stearothermophilus ATCC12016 glucosidase releases α–glucose residues by cleaving α–1-4-bonds successively from the non-reducing ends of maltosaccharides and soluble starch[2]. The similar type of enzyme was obtained from *Bacillus* sp. MIB010 and its phenotypic properties strikingly resembled to ATCC strain[3].

The present paper represents an analysis of the glucosidase activity by truncating the C-terminus ends of the enzyme molecule.

Materials and methods

Characterization of organisms

Phenotypic characterization was performed according to the methods described earlier[2].

Host and plasmids

Escherichia coli HB101 was used as a host for gene subcloning and expression. Vector plasmids used were pUC19[4]. The hybrid plasmid (named as pBST) carried the *Bacillus stearothermophilus* MIB010 α-glucosidase gene within a 2.8-kb *Sma*I fragment of DNA that had been inserted into pUC 19. *E. coli* cells were aerobically cultivated on L-broth medium containing 1% peptone, 0.5% NaCl, 0.5% yeast extract, 0.1% glucose, 50 μg/ml ampicillin and distilled water (pH 7.2).

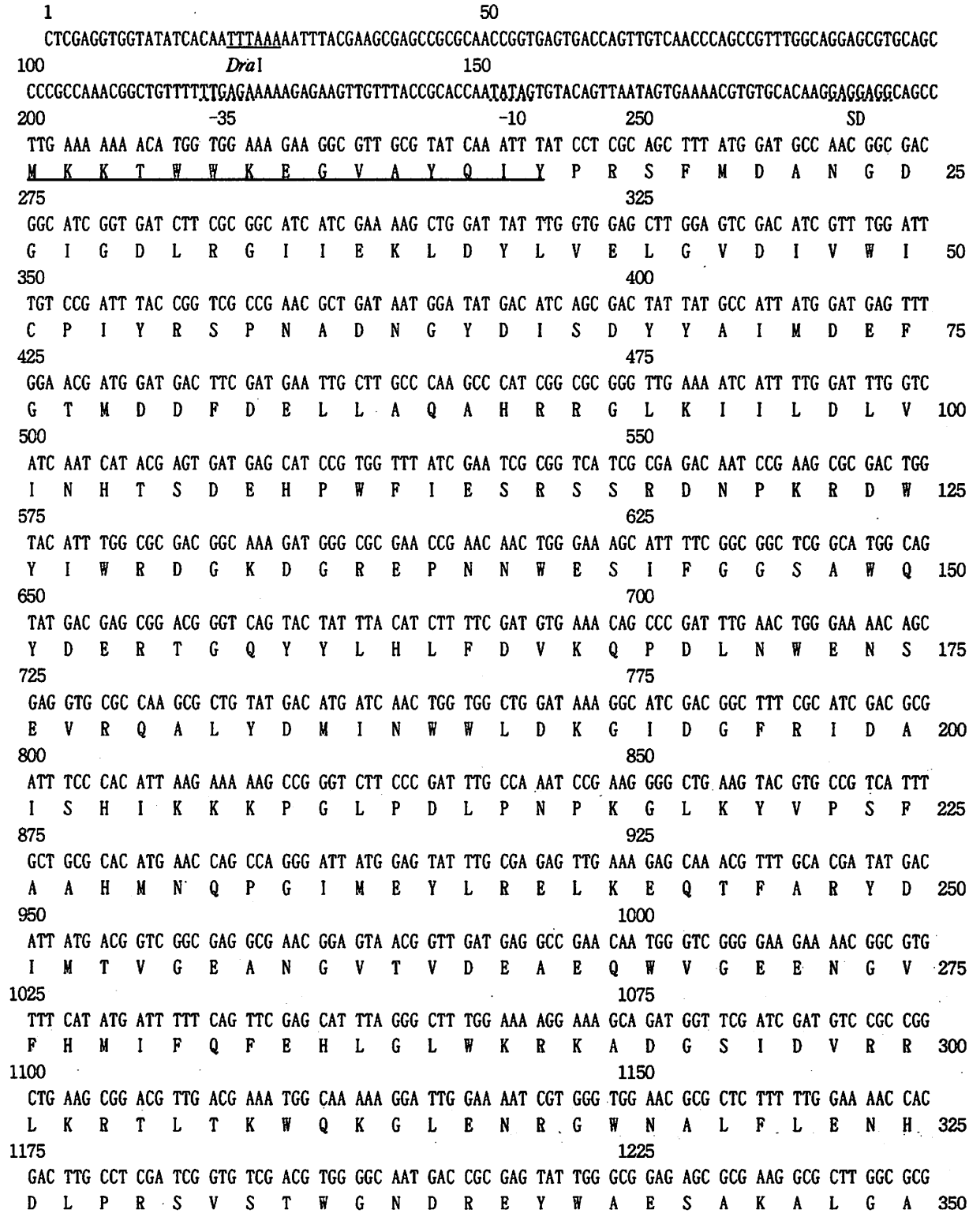
Plasmid subcloning

The appropriately shortened DNA fragments to be assayed were cloned within pUC19 in *E. coli* HB101. These fragments were generated by digesting the 2.8-kb fragment on pBST with restriction endonucleases, *Dra*I and *Pvu* II [named as pBST(DP)], or by treating the DNA fragments on pBST(DP) with the kilo-sequencing deletion kit (Takara Shuzo Co., Kyoto). The enzyme activity

expressed in *E. coli* cells were assayed by using *p*NPG as substrate according to the method of Paoni and Arroyo[5]. All other DNA manipulations were carried out as described by Sambrook et al[6].

Sequencing

DNA sequencing was conducted according to the dideoxy chain termination method[7], using M13 sequencing kit (Takara Shuzo, Kyoto).



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1250                                                                1300
  CTC TAC TTT TTC ATG CAA GGG ACG CCG TTC ATT TAC CAA GGG CAA GAG ATC GGG ATG ACG AAC GTG CAA TTC TCC
  L   Y   F   F   M   Q   G   T   P   F   I   Y   Q   G   Q   E   I   G   M   T   N   V   Q   F   S  375
1325                                                                1375
  GAC ATT CGC GAT TAC CGC GAT GTC GCT GCC TTG CGT CTG TAT GAG CTC GAA CGG GCG AAC GGC CGG ACG CAT GAG
  D   I   R   D   Y   R   D   V   A   A   L   R   L   Y   E   L   E   R   A   N   G   R   T   H   E  400
1400                                                                1450
  GAA GTG ATG AAG ATC ATT TGG AAA ACC GGG CGC GAC AAC TCG CGC ACC CCG ATG CAA TGG TCT GAT GCC CCG AAT
  E   V   M   K   I   I   W   K   T   G   R   D   N   S   R   T   P   M   Q   W   S   D   A   P   N  425
1475                                                                1525
  GCA GGG TTT ACG ACT GGC ACG CCA TGG ATC AAG GTG AAC GAA AAC TAT CGT ACG ATC AAT GTC GAG GCC GAG CGG
  A   G   F   T   T   G   T   P   W   I   K   V   N   E   N   Y   R   T   I   N   V   E   A   E   R  450
1550                                                                1600
  CGC GAC CCG AAC TCA GTA TGG TCG TTT TAT CGA CAA ATG ATT CAG CTT CGG AAA GCG AAC GAG CTG TTT GTT TAC
  R   D   P   N   S   V   W   S   F   Y   R   Q   M   I   Q   L   R   K   A   N   E   L   F   V   Y  475
1625                                                                1675
  GGA GCG TAC GAT CTG CTT TTG GAA AAT CAC CCA TCC ATT TAC GCG TAC ACA AGA ACG CTT GGC CGC GAT CGG GCG
  G   A   Y   D   L   L   L   E   N   H   P   S   I   Y   A   Y   T   R   T   L   G   R   D   R   A  500
1700                                                                1750
  CTT ATC ATT GTC AAC GTA TCC GAT CGT CCT TCA CTT TAC CGC TAT GAC GGC TTC CGC CTT CAG TCA AGC GAT TTG
  L   I   I   V   N   V   S   D   R   P   S   L   Y   R   Y   D   G   F   R   L   Q   S   S   D   L  525
1775                                                                1825
  GCG CTT TCG AAC TAC CCG GTC CGT CCG CAT AAA AAT GCG ACG CGT TTT AAG CTG AAG CCG TAC GAG GCG CGC GTA
  A   L   S   N   Y   P   V   R   P   H   K   N   A   T   R   F   K   L   K   P   Y   E   A   R   V  550
1850                                                          1900
  TAC ATC TGG AAA GAA TAAGAAGGAATTTGCCTTTCCGTGTGGAATAAATGCATACGGAAGGGAGATGATCAACATGGCAACGGTCGATCCGATT
  Y   I   W   K   E   ***
      1950                                                    2000
  CGCTACCCGATCGGAACGTTTCAAGCGCCGCAGCAGTTTGAGGCTGGGGAGGTGCAAGAGTGGATTGCTGCCATCCGCGGGTTGCCGAGCGACTTAAGA
      2050
  CCGCTGTTTCCGGCTTGAACGATGAGCAGCTG
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Fig. 1. The DNA sequence of the 2.1- kb DNA insert bearing the *B. stearothermophilus* MIB010 α-glucosidase gene and the deduced amino acid sequence of the enzyme.

Nucleotides of the 2.1-kb *Xho*I-*Pvu*II fragment (2075 bp) of MIB010 glucosidase gene are numbered consecutively in the direction from the 5'-terminus of the *Xho*I restriction site of the region. The underlined *Dra*I recognition site (TTTAAA) is located at positions 21-26. The SD sequence underlined corresponds to the putative ribosome-binding sequence; the –35 and –10 sequences underlined denote the transcriptional promoter sequences (–35 and –10) for *E.coli* RNA polymerase. The N-terminal sequences of 15 residues underlined agreed with that determined directly by a protein sequenator with the cloned α–glucosidase. The deduced amino acid sequence of the enzyme is indicated below the nucleotide triplets, with the residue numbers in the right column.

Enzyme assay

Glucosidase activity (designed as *p*NPGase) was assayed by the spectrophotometric method, as reported previously[4]. One unit (U) of the enzyme activity was defined as the amount of enzyme hydrolyzing 1 μmol substrate for 1 min at 60℃ and at pH 6.8. Protein was determine by the method of Lowry et al[8] with bovine serum albumin as the standard.

Molecular weight determination

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was run according to the methods of Laemni[9]. The molecular weights of proteins were determined by Western blotting technique according to the methods of Towbin et al[10], in which proteins were electrophoretically transferred to a nitrocellulose sheet (Millipore Corp., USA) that was layered on SDS gel. The nitrocellulose sheet was treated sequentially with rabbit anti-glucosidase antiserum (1:800), goat anti-rabbit immunoglobulin (1:100; Cappel Laboratories, USA) and rabbit anti-horseradish-peroxidase -horseradish peroxidase complex (1:250;Cappel), then stained with 3,3'-diaminobenzidine in the presence of H_2O_2. Standard molecular weight proteins (molecular weight, 14,000 – 92000; Bio-Rad Laboratories,USA) were transferred to the nitrocellulose sheet and then stained with 10% amido-Black.

Results and discussion

Characterization of Bacillus species.

Taxonomic and phenotypic properties did not distinguish *B. stearothermophilus* ATCC and MIB strains. *B.* sp. MIB010 has been tentatively assigned to a strain of *B. stearothermophilus* according to the method of Gibson and Gordon[11].

Nucleotide sequence

The *B. stearothermophilus* MIB010 glucosidase gene was cloned within a 2.1-kb *Dra*I-*Pvu*II fragment on pBST(DP). The fragment was composed of 2075 bp (Fig. 1), and the gene contained an open reading frame of 1665 bp. The frame initiated with a TTG start codon at nucleotide positions 199-201, and it terminated with a TAA stop codon at positions1865-1867.

The TTG start codon for Met in the present study is not rare since it has been found and recognized in other genes [12].

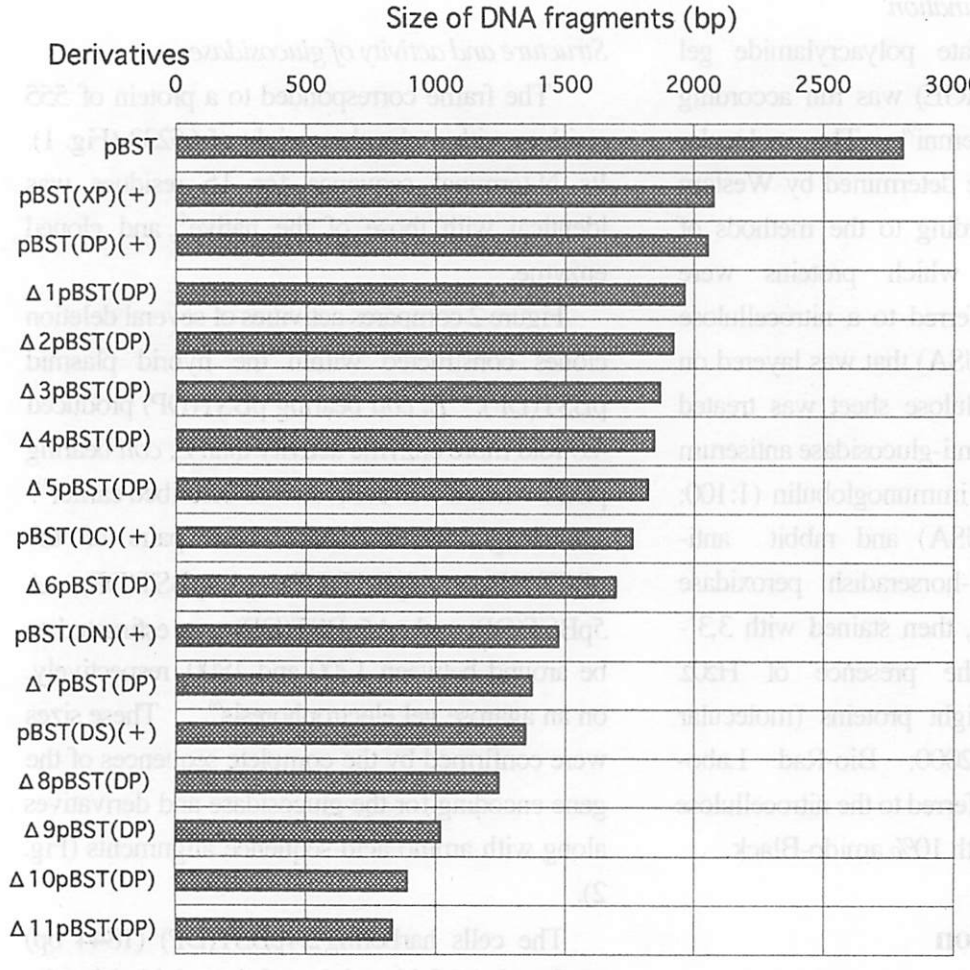
Structure and activity of glucosidase

The frame corresponded to a protein of 555 residues with molecular weight of 65233 (Fig. 1). Its N-terminal sequence for 15 residues was identical with those of the native[4] and cloned enzyme.

Figure 2 compares activities of several deletion clones constructed within the hybrid plasmid pBST(DP). *E. coli* bearing pBST(DP) produced 4.8-fold more enzyme activity than *E. coli* bearing pBST when it was cultivated as described earlier[3]. The length of nucleotide base pairs of the pBST(DP), Δ *1*pBST(DP), Δ *4*pBST(DP), Δ *5*pBST(DP) and Δ*6*pBST(DP) was estimated to be around between 1700 and 1800, respectively, on an agarose gel electrophoresis[6]. These sizes were confirmed by the complete sequences of the gene encoding for the glucosidase and derivatives along with amino acid sequence alignments (Fig. 2).

The cells harboring Δ*4*pBST(DP) (1844 bp) produced 4.4-fold activity of that yielded by the cells transformed with pBST(DP) (Fig. 2). However, only 10% of the enzyme activity were detected in the cells transformed by the plasmid Δ*5* pBST(DP)(1823 bp), which was truncated 21 bases from 3'-terminal of Δ*4*pBST(DP)[9]. Not any activity was detected in the *E. coli* cells, which was transformed by one of the plasmids:pBST (DC)(+) (1759 bp) and Δ*6*pBST(DP) (1695 bp), respectively.

Each of the mutated enzymes derived from pBST(DP), Δ 4pBST(DP), Δ 5pBST(DP) and pBST(DC)(+), respectively, gave single protein band with the similar molecular weights of approximately 65,000 on SDS gel, showing that all these molecules had the same antigen determinants in common (Fig. 3). Furthermore, these mutants exhibited considerable amounts (3.3 – 4.4 U/mg protein) of glucosidase activities (Fig. 2). Consequently, the plasmid Δ 4pBST(DP) presumably contains the minimum length (1844 bp) of glucosidase gene, which was sufficient for full enzymatic activity and stability, since its molecular weight was the same as those of strains from original MIB, ATCC, and cloned cells bearing whole 2.1-kb gene , respectively (Fig. 3).



final pH	Specific enzyme activity (U/mg protein)
8.5	0.910
8.5	4.355
8.6	3.456
8.6	4.025
8.6	3.180
8.5	3.302
8.5	3.448
8.5	0.399
8.3	0.000
8.4	0.000
8.4	0.000
8.3	0.000
8.2	0.000
8.5	0.000
8.6	0.000
8.6	0.000
8.6	0.000

Fig. 2. Comparison of glucosidase activity between wild strain (non-mutant) and several deleted mutants derived from *B. stearothermophilus* MIB010.

Nucleotides of the 2.1-kb *Dra*I-*Pvu*II fragment (2075 bp) of MIB010 glucosidase gene are truncated from the 3'-terminus of the *Pvu*II restriction site end. Each of the length of DNA fragments thus obtained is noted as base pairs (bp) from the *Xho*I 5'-terminus end of pBST. Δ1-11 means deletion mutants along with the size of the base pairs. C,D, N, P, S, X: restriction endonucleases; ClaI, *Dra*I, *Nru*I, *Pvu*II, *Sca*I and *Xho*I. (+): direction from 5'-terminus to 3'-terminus end. Final pH denotes pH value of culture broth of the cloned strains.

Each of the mutated enzymes derived from pBST(DP), Δ *4*pBST(DP), Δ *5*pBST(DP) and pBST(DC)(+), respectively, gave single protein band with the similar molecular weights of approximately 65,000 on SDS gel, showing that all these molecules had the same antigen determinants in common (Fig. 3). Furthermore, these mutants exhibited considerable amounts (3.3 – 4.4 U/mg protein) of glucosidase activities (Fig. 2). Consequently, the plasmid Δ *4*pBST(DP) presumably contains the minimum length (1844 bp) of glucosidase gene, which was sufficient for full enzymatic activity and stability, since its molecular weight was the same as those of strains from original MIB, ATCC, and cloned cells bearing whole 2.1-kb gene , respectively (Fig. 3).

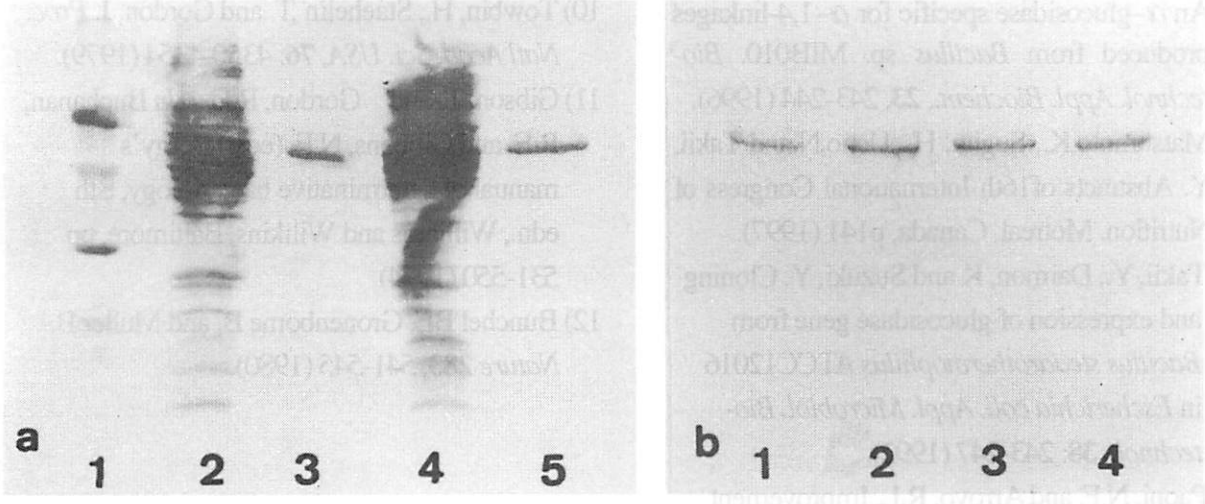


Fig. 3. The SDS-PAGE of glucosidase from non-mutant and deleted mutants bearing *B. stearothermophilus* MIB010 α -glucosidase gene.

The electrophoresis was carried out as described in the text. The proteins in each gel were transferred to nitrocellulose sheet. In *panel a*, proteins transferred were stained with amido black. Lane 1: standard molecular weight proteins (67,000, 46,000, 31,000 and 21,000); Lane 2: cell-free extract of *E. coli* bearing pBST(DP); Lane 3: purified glucosidase (1.2 μg) of *B. stearothermophilus* MIB010; Lane 4: cell-free extract (3.5 μg) of *E. coli* bearing Δ*4*pBST(DP); Lane 5: pure preparation of *B. stearothermophilus* ATCC12016 (0.5 μg). In panel b, proteins transferred were subjected to the immunodetection, using specific rabbit antiserum prepared against *B. stearothermophilus* MIB010 glucosidase as primary antibody, as described in the text. The antiserum was found to precipitate 0.2 U of purified MIB 010 enzyme[3]. Lane 1: cell-free extract (3.8 μg) of *E. coli* bearing pBST(DP); Lane 2; cell-free extract (5.5 μg) of *E. coli* bearing Δ*4*pBST(DP); Lane 3: cell-free extract (2.1 μg) of *E. coli* bearing Δ*5*pBST(DP); Lane 4: cell-free extract(1.5 μg) of *E. coli* bearingpBST(DC)(+), respectively.

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