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Cloning and Characterization of α-Glucosidase Gene from Thermophilic *Bacillus* sp. DG0303

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Abstract An α-glucosidase gene (*aglA*) from thermophilic *Bacillus* sp. DG0303 was cloned, sequenced, and expressed in *Escherichia coli*. The *aglA* was localized to the 2.1-kb *Pvu*I-*Xmn*I region within the 5.9-kb DNA insert of the hybrid plasmid pAG1. The gene consisted of an open reading frame of 1,686 bp with an unusual GTG initiation codon and TGA termination codon. The amino acid sequence deduced from the nucleotide sequence predicted a protein of 562 amino acid residues with a M_r of 66,551 dalton. A comparative amino acid sequence analysis revealed that DG0303 α-glucosidase is related to bacillary oligo-1.6-glucosidases. The *Bacillus* sp. DG0303 α-glucosidase showed a high sequence identity (36-59%) to the *B. flavocaldarius*, *B. cereus*, and *B. thermoglucosidasius* oligo-1,6-glucosidases. The number of prolines in these four α-glucosidases was observed to increase with increasing thermostability of these enzymes. The cloned α-glucosidase was purified from *E. coli* DH5α bearing pAG1 and characterized. The recombinant enzyme was identical with the native enzyme in its optimum pH and in its molecular mass, estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The temperature optimum of the cloned α-glucosidase was lower than that of the native enzyme.

Key words: α-Glucosidase. oligo-1.6-glucosidase, gene cloning, nucleotide sequence, expression

α-Glucosidases hydrolyze the terminal nonreducing α-D-glucosidic linkage of oligo- and polysaccharides with the release of α-glucose. α-Glucosidases are produced by many mesophilic and thermophilic bacteria [8]. The substrate specificity of α-glucosidases differ greatly depending on the source of the enzymes, and two types of activities of bacterial α-glucosidases have been reported: α-glucosidase (EC 3.2.1.20, α-D-glucoside glucohydrolase) and oligo-1,6-α-glucosidase (EC 3.2.1.10, dextrin 6-α-D-glucanohydrolase) [17]. These enzymes have a number of potential applications as they exhibit a wide range of substrate specificities and transglucosylation activities [8].

Interest in thermostable enzymes has grown, mainly due to the fact that most existing industrial enzyme processes are carried out at high temperatures [5, 9, 22]. There are several advantages in using thermostable enzymes in industrial processes as compared to thermolabile enzymes. The main advantage is that as the temperature of the process is increased, the rate of reaction increases [24]. Thermostable enzymes have longer half-lifes. The use of higher temperatures also is inhibitory to microbial growth, thereby decreasing the possibility of microbial contamination.

So far, the thermostability of α-glucosidases has been analyzed mainly in terms of their amino acid compositions. Suzuki proposed a general rule for protein thermostability, the 'proline theory', based on the strong correlation observed between the thermostability and proline content of several *Bacillus* oligo-1.6-glucosidases [21]. This theory suggests that a protein is thermostabilized by increasing the frequency of the proline occurrence at β-turns, along with the total number of hydrophobic residues present in the protein.

The thermophilic strain *Bacillus* sp. DG0303 produces a thermostable α-glucosidase activity [16]. Accordingly. a comparison of the primary structure of this α-glucosidase with those of other α-glucosidases with different thermostabilities should provide important information on the structure-function relationship of α-glucosidases. In the present study, as a first step towards confirming the above proline hypothesis, the α-glucosidase gene from *Bacillus* sp. DG0303 was cloned, sequenced, and expressed in *E. coli*.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media

The chromosomal DNA of *Bacillus* sp. DG0303 [16] was used as the source of the α-glucosidase gene. *Escherichia coli* strain DH5α [19] was used as the host for the gene cloning and expression. Plasmid pUC118 [23] was used as

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the cloning vector. *E. coli* DH5α was grown at 37°C in either an LB medium (10 g of tryptone, 5 g of yeast extract, 5 g of NaCl per liter) or LB supplemented with ampicillin (50 μg/ml) when selecting the transformants. *Bacillus* sp. DG0303 was grown in a nutrient broth (Difco, Detroit, MI, U.S.A.) at 55°C.

Genomic Library Construction and Screening of α-Glucosidase-Positive Clone

The chromosomal DNA of *Bacillus* sp. DG0303 was prepared using the method of Marmur [14]. The genomic DNA was partially digested with the restriction enzyme *Sau*3AI, and DNA fragments of 3 to 10 kilobases (kb) were then recovered from an agarose gel using an Elutrap™ apparatus (Schleicher and Schuell, Keene, U.S.A.) according to the manufacturer's instructions. The electroeluted DNA was ligated with T4 DNA ligase into *Bam*HI-digested, dephosphorylated pUC118, and the ligation mixture was transformed into competent *E. coli* DH5α. The white colonies which grew on an L-broth agar plate containing 50 μg/ml ampicillin, 1 mM isopropyl-β-thiogalactoside (IPTG), and 0.02% 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) were screened for thermostable α-glucosidase activity using an enzymatic assay at 55°C on an agar plate with 4-methylumbelliferyl-α-D-glucoside (α-MUG), a synthetic substrate for α-glucosidase. The colonies which possessed α-glucosidase activity exhibited fluorescence under UV light due to the production of 4-methylumbelliferone. The positive clone carried a hybrid plasmid, named pAG1, containing a 5.9-kb DNA insert in pUC118.

Restriction Enzyme Mapping and Deletion Analysis

The DNA was digested with various restriction endonucleases, electrophoresed through agarose gels in 45 mM Tris-45 mM boric acid-1 mM EDTA (TBE), and stained with ethidium bromide. A deletion analysis of the cloned DNA fragment was performed using an Erase-a-Base® System (Promega, Madison, U.S.A.) according to the manufacturer's instruction.

DNA Sequencing

Nucleotide sequencing was carried out on both strands using the dideoxy-chain termination method [20] with a SILVER SEQUENCE™ DNA Sequencing System (Promega). Sequence analyses were accomplished with the DNA Strider program (version 1.1). The BLAST program (version 2.0) [2] was used to search the protein sequence database at the National Center for Biotechnology Information (National Institutes of Health Bethesda, MD, U.S.A.).

Purification of Cloned α-Glucosidase

All chromatographic steps were performed on a ProTeam™ LC system 210 (Isco Inc., Lincoln, U.S.A.) at room temperature, and the purification steps were monitored by activity measurements with the assay described below. *E. coli* DH5α bearing pAG1 was grown at 37°C overnight in an LB broth containing 50 μg/ml ampicillin. The cells (from a 2-l culture) collected by centrifugation were disrupted in buffer A (50 mM sodium acetate buffer, pH 5.0) by sonication, and centrifuged to remove any cell debris. The sonicated extract was treated at 60°C for 15 min and centrifuged to remove any denatured proteins. The heat-treated sample was adjusted to 1 M ammonium sulfate and put on a Butyl-Sepharose column (Pharmacia, Uppsala, Sweden) equilibrated with buffer A containing 1 M ammonium sulfate. The elution was with a linear gradient of 1.0–0 M ammonium sulfate. The fractions containing α-glucosidase activity were pooled and dialyzed against buffer A. The dialyzate was concentrated by ultrafiltration using a 10-kDa-cutoff membrane (PM10; Amicon, Beverly, U.S.A.), and applied to a Q-Sepharose column (2.5 cm×20 cm) equilibrated with buffer A. The α-glucosidase activity was eluted using a linear gradient of 0–1.0 M NaCl at a flow rate of 0.5 ml/min, and the active fractions were pooled and concentrated. The concentrate was put on a Sepharose 6B gel filtration column (1.0× 60 cm) equilibrated with buffer A containing 200 mM NaCl. The column was eluted at a flow rate of 0.2 ml/min.

α-Glucosidase Assay and Protein Determination

The α-glucosidase activity was determined by measuring the *p*-nitrophenol (*p*NP) released from *p*-nitrophenyl-α-D-glucoside (*p*NPG; Sigma Chemical Co., U.S.A.) as described previously [16]. One unit of enzyme activity was defined as the amount of enzyme that liberated 1 μmol of *p*NP per min. The protein concentrations were determined as described by Bradford [3], with bovine serum albumin as the standard. SDS-PAGE was performed with a 12% acrylamide, and the gel was stained with Coomassie brilliant blue R-250 [11].

Nucleotide Sequence Accession Number

The nucleotide sequence of the α-glucosidase gene (*aglA*) from *Bacillus* sp. DG0303 was submitted to the GenBank under accession no. AF216220.

RESULTS

Cloning of α-Glucosidase Gene from *Bacillus* sp. DG0303

A genomic library was constructed from *Bacillus* sp. DG0303 from which the thermostable α-glucosidase had previously been isolated and characterized [16]. About 1,500 ampicillin-resistant recombinants were screened for α-glucosidase activity using the synthetic substrate α-MUG. This method detected only those colonies which

possessed α-glucosidase activity and *E. coli* DH5α did not show any fluorescence using this method. One clone showed fluorescence under UV light, after incubation for 4 h at 55°C, indicating the liberation of 4-methylumbelliferone. Overnight incubation at temperatures higher than 55°C was not applied due to the thermal lability of the substrate. The positive clone was purified three times by single colony isolation prior to plasmid DNA extraction. The hybrid plasmid containing the α-glucosidase gene in this clone was designated as pAG1.

Restriction Mapping and Deletion Analysis

A restriction endonuclease analysis of pAG1 on agarose gel electrophoresis revealed the insertion of a 5.9-kb fragment of DNA within pUC118. The insert isolated from the gel was treated with various endonucleases, and a restriction map was constructed, as shown in Fig. 1. A series of nested deletions of the 5.9-kb DNA insert was constructed with exonuclease III to localize the α-glucosidase gene and it was found to be within a 2.1-kb *Pvu*I-*Xmn*I region (data not shown).

Sequence Analysis of α-Glucosidase Gene

Figure 2 shows the nucleotide sequence of the 2.1-kb *Pvu*I-*Xmn*I DNA fragment and the deduced amino acid sequence of *aglA*. Additional upstream and downstream sequences are also presented. The *aglA* gene was comprised of a coding sequence of 1,686 nucleotides beginning with an unusual GTG initiation codon at nucleotide positions 226–228 and terminating with a TGA (stop) codon at nucleotide positions 1912–1914. This open reading frame (ORF) encoded a polypeptide of 562 amino acid residues with a calculated molecular mass (M_r) of 66,551. The M_r was fairly comparable with that of the native α-glucosidase purified from *Bacillus* sp. DG0303 [16]. The unusual GTG initiation codon has been previously reported in several bacterial genes, such as those for *Ampullariella* sp. xylose isomerase [18], *Pseudomonas putida* histidine

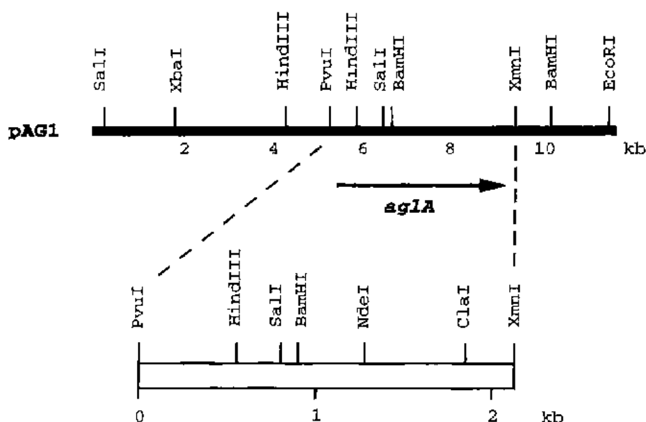

Fig. 1. Restriction map of DNA fragment containing the α-glucosidase gene (*aglA*) of *Bacillus* sp. DG0303.

The 5.9-kb *Sau*3AI DNA insert within the hybrid plasmid pAG1 is shown by the solid bar. An arrow (*aglA*) indicates the direction and extent of the α-glucosidase gene. The open box represents the 2.1-kb *Pvu*I-*Xmn*I region of pAG1 containing the α-glucosidase gene. The vector pUC118 region within pAG1 is omitted from the figure.

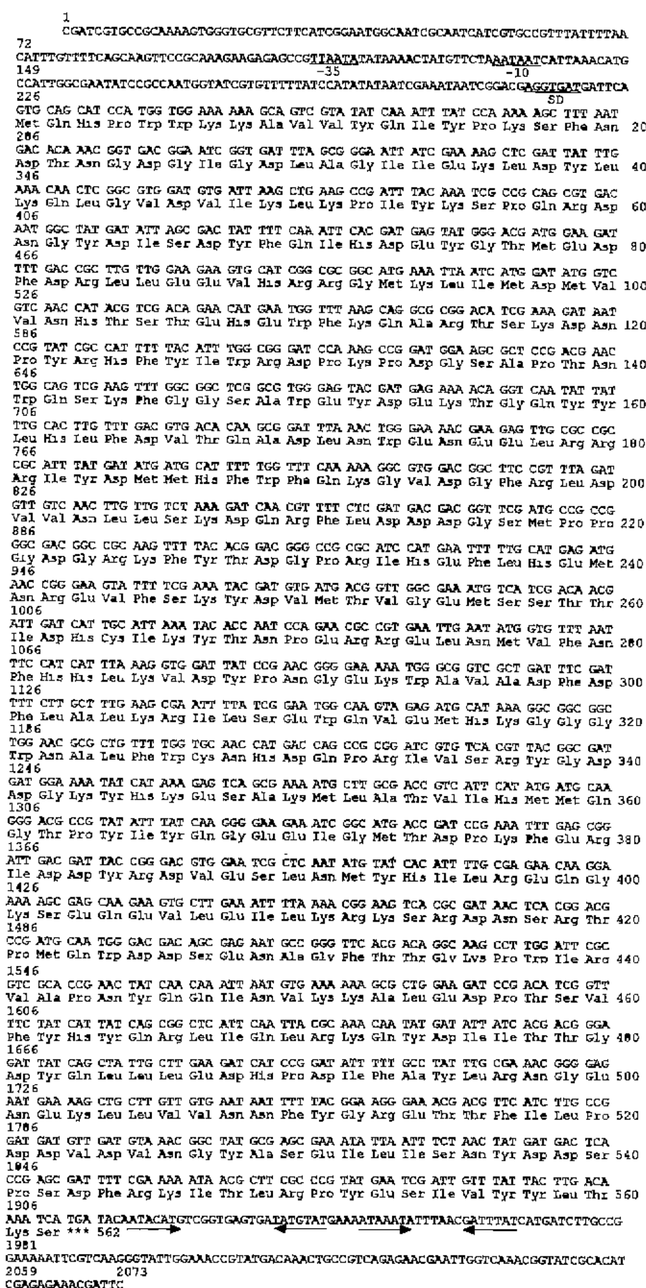

Fig. 2. Nucleotide sequence of the α-glucosidase gene of *Bacillus* sp. DG0303 and deduced amino acid sequence.

The nucleotides of the 2.1-kb *Pvu*I-*Xmn*I DNA fragment are numbered from the 5'-terminus of the *Pvu*I restriction site. The putative Shine-Dalgarno ribosome-binding sequence (SD) and the promoter -10 and -35 regions are underlined. The two inverted repeats for the transcriptional termination are indicated with arrows. The underlined amino acid sequences were previously determined with α-glucosidase purified from *Bacillus* sp. DG0303.

ammonia-lyase [4], *Thermus aquaticus* carboxypeptidase [12], and *Bacillus psychrosaccharolyticus* alanine racemase [15]. The *aglA* gene was preceded by a potential ribosome-binding site (AGGTGAT), centered 10 nucleotides from the initiation codon. The nucleotides that may represent the -10 and -35 regions of the putative *aglA* promoter are shown in Fig. 2. Two inverted repeats occurred at 3'-flanking downstream of the open reading frame. These inverted repeats would produce two potential hairpin structures in the resultant mRNA, if transcribed. The first (positions 1919-1943) had 13 nucleotides in its loop and 6 bp in its stem, and the second (positions 1448-1967) was comprised of 8 nucleotides in the loop and 6 bp in the stem. These structures were not followed by any successive run of thymidine residues. These features could facilitate a typical ρ-dependent transcription termination [1].

In an extensive database search for proteins with sequence similarities, the α-glucosidase of *Bacillus* sp. DG0303 showed a very high sequence identity to bacillary oligo-1,6-glucosidases. The DG0303 α-glucosidase had a 36%, 56%, and 59% sequence identity with the oligo-1,6-glucosidases from *B. flavocaldarius* [7], *B. cereus* [26], and *B. thermoglucosidasius* [25], respectively. The amino acid sequence alignment of the DG0303 α-glucosidase to these three oligo-1,6-glucosidases is shown in Fig. 3. The oligo-1,6-glucosidases from *B. cereus*, *B. thermoglucosidasius*, and *B. flavocaldarius* and *Bacillus* sp. DG0303 α-glucosidase contain 19, 32, 47, and 24 proline residues, respectively, which correspond to a proline content of 3.41, 5.69, 8.90, and 4.27 mol% (Fig. 3, Table 1). Thirteen of these occur at common sites in four of the enzymes, which refer to positions 16, 52, 57, 130, 137, 231, 331, 362, 421, 437, 443, 520, and 549 of the *B. cereus* enzyme (Fig. 3).

```
Bce  MEKQWWKESVVYQIYPRSFMDSNGDGIGDLRGIISKLDYLKELGIDVIWLSPVYESPNDD   60
Bth  **RV****A**********Y**********I****A**********V**V*******K*****   60
Bfl  *S—**QRA*I**V*****Q*T*****V***E**RRR*P***S**V*AL****F*K**MK*   58
Bsp  *QHP***KA********K**N*T*********A***E*******Q**V***K*K*I*K**QR*   60
       1          4                      1           4    4

Bce  NGYDISDYCKIMNEFGTMEDWDELLHEMHERNMKLMMDLVVNHTSDEHNWFIESRKSKDN  120
Bth  *********RD**D******A***KTM*E****K*GI**V*************P*************  120
Bfl  F***VA***DVDPV****LQ*F*R**E*A*ALGL*VLV***P*****S**P**L****A*RNS  118
Bsp  *********FQ*HD*Y******F*R**E*V*R*G****I**M*******T**E***KQA*T****  120
              1                           1       2

Bce  KYRDYYIWR-PGKEGKEPNNWGAAFSGSAWQYDEMTDEYYLHLFSKKQPDLNWDNEKVRQ  179
Bth  P*********-****N*********ESV*******E*****G*****************E*P****R  179
Bfl  PK**W****KD**APD*GP****QSF*G*P**TL***A*GQ*******LPE********R*PE**E  178
Bsp  P**HF****D*KPD*SA*T***QSK*G*****E****K*GQ*******DVT*A*****E**EL*R  180
     3        4 2   14          1                1  3      2

Bce  DVYEMMKFWLEKGIDGFRMDVINFISKEEGL——PTVETEEEG--YVSGH-K-HFMNGPN  232
Bth  E***********D***V************M****VPE*----*DG*-PQS*KK*A***S-R-YY*****R  233
Bfl  AIK*V*R****RR*V*****V***LWLLG*DPLFRDE*GSPLWRP*LPDRAR*EHLYTEDQ*E  238
Bsp  RI*D**H**FQ**V*****L***V*LL**DQRF-------LDDD*SMPPGDGR*-FYTD**R  232
                              2     3     1 1  111          4

Bce  -IHKYLHEMNEEVLSHY------D-IMTVGE-MPGVTTEEAKLYTGEERKELQMVFQFEHM  284
Bth  -V*EF*Q****R*****K*------*-*******-T*****PK*GI****DPS*R**N**********  285
Bfl  TY-A*VR***-RQ**DEFSEPGRERV*-****IYLPL-PRLVRY*AAG----CHLP*N*SLV  290
Bsp  -**EF******R**F*K*------*-V******-*SST*IDHCIK**NP**R**N****N*H*L  284
                      1           21  2         2       1

Bce  DLDSGEGGKWDVKPCSLLTLKENLTKWQKALEHTG-WNSLYWNNHDQPRVVSRFGNDGMY  343
Bth  *****P******IR*W***AD***KTM******E**GK*-*****L*********A******D**K*  344
Bfl  TE------*LS*WR*EN*ARI---VETYEGL*SRWD-*PNWVLG*******LA**L*E----  337
Bsp  KV*YPN*E***A*ADFDF*A***RI*SE***VEMHKG*G**A*F*C*******I****T*D**K*  344
        11      3                       1         4

Bce  RIESAKMLATVLHMMKGTPYIYQGEEIGMTNVRFESIDEYRDIETLNMYKEKVMERGEDI  403
Bth  *V**********F*****Q*************************P***ED***************R*E*Y****P  404
Bfl  P--Q*RVA*ML*FTLR****TW*Y*D*LALP*GLIP-PEKVQ*PAA*---R----Q*DREP  387
Bsp  HK***********I****Q*****************DPK***R***D****V*S*****-HILR*Q*KSE  403
     1                4           1 1  2 1      1                2

Bce  EKVMQSIYIKGRDNARTPMQWDDQNHAGFTTGEPWITVNPNYKEINVKQAIQNKDSIFYY  463
Bth  QE***EK***Y*****************SEN*****A*T****P************A*LEDPN*V*H*  464
Bfl  T-AYHTL---****PE*****P***ASPYG***S*V****LPL***D*RTR***AVQE*DPR*MLHL  443
Bsp  QE*LEILKR*S****S***********SEN********K****R*A****QQ*****K*LEDPT*V***H  463
                   1   4 1    1          4  2  4              3

Bce  YKKLIELRKNNEIVVYGSYDLILENNPSIFAYVRTYGVEKLLVIANFTAEECIFELPEDI  523
Bth  *****Q****QHD*I****T********DD*Y*YR*T***L*N*Q*I***T***SEKTPV*R***DH*  524
Bfl  V*R***A****-DPDLL***A*RT-YRAREGVY***L*—*-*GW***AL*L*EK*KAL****RG-  497
Bsp  *QR***Q****QYD*ITT*D*Q*L***DH*D*****L*NGEN*******VN***YRG*TT*I***D*V  523
               1              3                           1    4

Bce  SYSEV--ELLIHNYDV-ENGPIENITLRPYEAMVFK--L--K  558
Bth  I*KTK--*****S*****D*AEELKE*R****W***R*Y*IR*--P  562
Bfl  —GR*--V*-STHL*R-*ERVG*RLF****D*GVAVR--*--D  529
Bsp  DVNGYAS*I**S****D-SPSDFRK********SI*YY--*TKS  562
                  1        4            1
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Fig. 3. Comparison of amino acid sequence between α-glucosidase of *Bacillus* sp. DG0303 (*Bsp*) and oligo-1,6-glucosidases of *B. cereus* (*Bce*) [26], *B. thermoglucosidasius* (*Bth*) [25], and *B. flavocaldarius* (*Bfl*) [7].

The residues identical to those of the *B. cereus* enzyme are indicated by asterisks. Gaps (-) were introduced during the alignment. The numbers of proline occurrences are given under the primary sequences.

Purification and Characterization of Cloned α-Glucosidase

In order to study the cloned α-glucosidase, the α-glucosidase gene was expressed in *E. coli* DH5α cells, purified, and characterized. The cloned α-glucosidase was expressed constitutively during growth in an LB medium and produced as a cytoplasmic protein in *E. coli* DH5α bearing pAG1 (data not shown). The purification of the cloned enzyme involved the heat fractionation of the clarified cell sonicates followed by hydrophobic chromatography, anion-exchange chromatography, and gel filtration chromatography. Table 2 summarizes the purification of the α-glucosidase produced by *E. coli* DH5α bearing

Table 1. Comparison of α-glucosidase from *Bacillus* sp. DG0303 and oligo-1,6-glucosidases from *Bacillus* species in proline content and thermostability.

Bacillus species	Number of amino acid residues	Proline residues (mol%)	Optimum temperature (°C)	$T_{inact\ 50\%}$ [a] (°C)	References
B. cereus ATCC7064	558	19 (3.41)	40	44	[26]
Bacillus sp. DG0303	562	24 (4.27)	60	70	This study, [16]
B. thermoglucosidasius KP1006	562	32 (5.69)	60	72	[25]
B. flavocaldarius KP1228	529	47 (8.90)	87	89	[7]

[a] $T_{inact\ 50\%}$ is defined as the temperature that caused 50% inactivation when the purified enzymes were incubated for 10 min.

Table 2. Purification of α-glucosidase from *E. coli* DH5α bearing pAG1.

Purification step	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)
Cell extract	100	261,880	2,619	100
Heated extract	39	236,760	6,071	90
Butyl-Sepharose	23	184,870	8,038	71
Q-Sepharose	9	122,980	13,662	47
Sepharose 6B	2	98,750	49,375	38

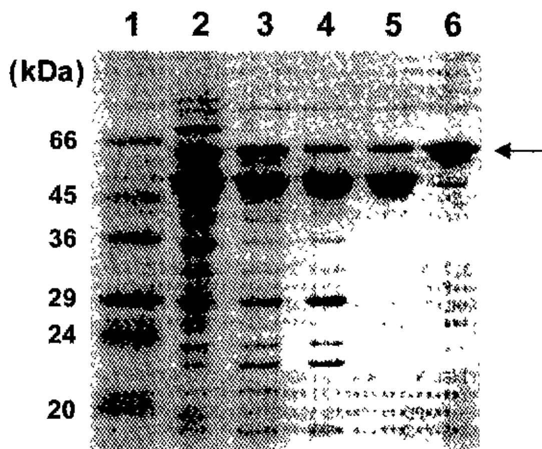


Fig. 4. SDS-PAGE of cloned α-glucosidase at various stages of purification.

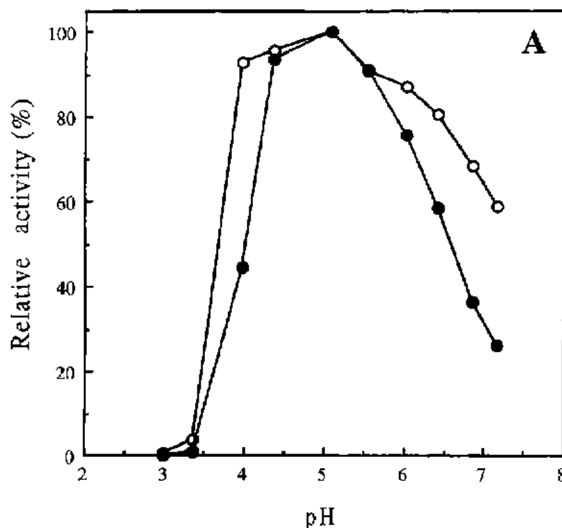
Lane 1, protein standards (Sigma), lane 2, sonicated extracts; lane 3, after heat treatment; lane 4, after Butyl-Sepharose, lane 5, after Q-Sepharose; lane 6, after Sepharose 6B. The position of the purified α-glucosidase is shown by an arrow The numbers on the left indicate the sizes of the protein standards.

pAG1. The final enzyme preparation was purified 19-fold compared with the cell extracts and the yield was 38%. The final preparation yielded a main protein band and a few faint bands on SDS-PAGE (Fig. 4). The M_r of the cloned enzyme was estimated to be 60 kDa which was identical to that of the native enzyme [16].

The pH and temperature dependencies of the native and cloned α-glucosidase activities were examined (Fig. 5). Both enzymes exhibited an identical pH optimum of 5.0 for a *p*NPG hydrolysis (Fig. 5A). In contrast to its identical molecular weight and pH optimum with the native enzyme, the optimal temperature of the cloned α-glucosidase was 15°C lower compared with that of the native enzyme, and the activity at 60°C was about 0.76-fold that at 45°C (Fig. 5B). At 45°C, the cloned α-glucosidase retained its initial activity during 2 h of incubation.

DISCUSSION

A large number of proteins were screened for their amino acid sequence similarity to *Bacillus* sp. DG0303 α-glucosidase, using an updated version of a protein sequence database. This computer search showed a high sequence similarity between *Bacillus* sp. DG0303 α-glucosidase and the oligo-1,6-glucosidases from several *Bacillus* species. A comparison of the deduced amino acid sequences of three bacillary oligo-1,6-glucosidases and DG0303 α-glucosidase showed a high sequence similarity between these enzymes (Fig. 3). The identification of *Bacillus* sp. DG0303 α-glucosidase as a oligo-1,6- glucosidase was also confirmed by its substrate specificity, as reported



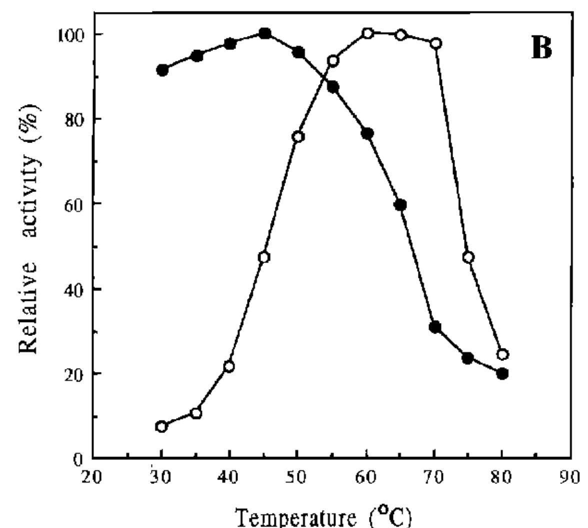


Fig. 5. Effects of pH (A) and temperature (B) on activities of native and cloned *Bacillus* sp. DG0303 α-glucosidases.

The activities of the native and cloned enzymes are indicated by open and closed circles, respectively. (A) The native and cloned enzymes were assayed at 60°C and 45°C, respectively The relative activities were determined at various pHs using a McIlvaine (citric acid-Na_2HPO_4) buffer. (B) The activity of each enzyme was assayed as described in Materials and Methods, except that the reaction temperatures ranged between 30 to 80°C.

previously [16]. Recently, the three-dimensional structure of *Bacillus cereus* ATCC7064 oligo-1,6-glucosidase was determined by X-ray crystallography [10]. The high sequence similarity of DG0303 α-glucosidase with the oligo-1,6-glucosidase of *B. cereus* would, therefore, suggest that the folded conformation of the DG0303 enzyme is similar to that of the *B. cereus* oligo-1,6-glucosidase. *Bacillus* sp. DG0303 α-glucosidase and other oligo-1,6-glucosidases contain sequences corresponding to the secondary elements revealed in the *B. cereus* oligo-1,6-glucosidase [27]. Based on secondary-structural analyses, these oligo-1,6-glucosidases have recently been suggested to have an α-amylase-type $(\alpha/\beta)_8$-barrel (or TIM barrel) structure [6, 13]. Thus, it would appear that the DG0303 α-glucosidase can be assigned as a member of the 'α-amylase' family.

The thermostability and thermal optimum of the cloned α-glucosidase were lower than those of the native enzyme. Although the reason for this is not clear, it would seem that the conformation of the cloned enzyme changed a little in comparison with that of the native enzyme. The alteration of the enzyme's thermal stability may be due to uncorrected folding of the thermostable protein in the case of its expression in mesophilic bacterial strains.

The intrinsic basis of protein thermostability is not well understood so far. It has been postulated that the thermal adaptation of a protein is accomplished by the cumulation of many small stabilization effects on its peripheral neutral regions, resulting in the molecule being more packed [22]. The proline theory based on a comparative analysis of various bacillary oligo-1,6-glucosidases has also been proposed as a general rule for protein thermostability [21]. Proline residues critical for thermal stabilization favor the second sites of β turns and N-caps of α helices [27]. A comparison of the proline contents and temperatures for a 50% inactivation of the DG0303 α-glucosidase and three bacillary oligo-1,6-glucosidases indicated a strong correlation between the increase in the proline contents and the increase in their thermostability of these glucosidases (Table 1). Comparative X-ray crystallographic and site-directed mutagenesis studies could provide the more definitive information needed to confirm the 'proline theory'.

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