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Cloning and Expression of Thermostable β-Glycosidase Gene from *Thermus filiformis* Wai33 A1 in *Escherichia coli* and Enzyme Characterization

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Abstract A thermostable β-glycosidase gene, *tfi β-gly*, was cloned from the genomic library of *Thermus filiformis* Wai33 A1. *tfi β-gly* consists of 1,296 bp nucleotide sequence and encodes a polypeptide of 431 amino acids. It shares a strong amino acid sequence similarity with the β-glycosidases from other *Thermus* spp. belonging to the glycosyl hydrolase family 1. In the present study, the enzyme was overexpressed in *Escherichia coli* BL21 (DE3) using the pET21b(+) vector system. The recombinant enzyme was purified to homogeneity by heat treatment and a Ni^{2+}-affinity chromatography. Polyacrylamide gel electrophoresis (PAGE) showed that the recombinant Tfi β-glycosidase was a monomeric form with molecular mass of 49 kDa. The temperature and pH range for optimal activity of the purified enzyme were 80–90°C and 5.0–6.0, respectively. Ninety-three percent of the enzyme activity was remained at 70°C after 12 h, and its half-life at 80°C was 6 h, indicating that Tfi β-glycosidase is highly thermostable. Based on its K_m or K_{cat}/K_m ratio, Tfi β-glycosidase appeared to have higher affinity for β-D-glucoside than for β-D-galactoside, however, K_{cat} for β-D-galactoside was much higher than that for β-D-glucoside. The activity for lactose hydrolysis was proportionally increased at 70°C and pH 7.0 without substrate inhibition until reaching 250 mM lactose concentration. The specific activity of Tfi β-glycosidase on 138 mM lactose at 70°C and pH 7.0 was 134.9 U/mg. Consequently, this newly cloned enzyme appears to have a valuable advantage of conducting biotechnological processes at elevated temperature during milk pasteurization in the production of low-lactose milk.

Key words: β-Glycosidase, *Thermus filiformis* Wai33 A1, lactose hydrolysis, low-lactose milk

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Lactase deficiency is one of the most common genetic disorders afflicting more than 50% of the world's population and often causes lactose intolerance [6]. Lactose-intolerant individuals are unable to digest significant amount of lactose in milk because of lactase deficiency and, on ingestion of milk or dairy products, may undergo many clinical symptoms such as bloating, abdominal cramps, flatulence, and diarrhea [29]. Lactose intolerance is associated with low milk consumption, resulting in depression of the dairy industry in many Asian and African countries. Lactose intolerance, however, can be overcome by introducing an enzymatic hydrolysis of lactose during the process of milk and diary products. β-Galactosidase (EC 3.2.1.23) is the enzyme which catalyzes the hydrolysis of β-1,4-D-galactosidic linkage of lactose to its component monosaccharides, glucose and galactose. Therefore, β-galactosidases from various sources have been investigated and some enzymes have already been utilized in large-scale processes [8, 23].

In addition to β-galactosidase, β-glycosidase (EC 3.2.1.21), a key enzyme for carbohydrate metabolism in Archaea, Bacteria, and Eukarya, is also involved in hydrolysis of lactose. Due to its broad substrate specificity, β-glycosidase is one of the useful tools in many applications, including the dairy industry [11, 12, 20].

Thermostability of the enzyme allows to process at high temperature in industrial applications. There are several valuable advantages in conducting the processes at elevated temperature: It reduces the risk of contamination by common mesophiles and attains higher reaction rates due to a decrease in viscosity and an increase in diffusion coefficiency of substrates, eventually resulting in down-cost during processing [22].

Thermus spp., the most commonly investigated thermophilic eubacteria, are known to produce thermostable β-glycosidase

or β-galactosidase. Since the optimal conditions for cultivating *Thermus* spp. approximate well with milk pasteurization conditions (pH 7.0 and 70°C), we have attempted to clone thermostable lactose hydrolase from one of the *Thermus* strains that would be suitable for use in the dairy industry, especially low-lactose milk production. Recently, thermostable β-glycosidases have been cloned and characterized from *T. thermophilus* HB27 [5], *T. caldophilus* GK24 [9], and *T. nonproteolyticus* HG102 [10]. Although some enzymatic properties of β-glycosidases from *Thermus* spp. were discussed in those papers mentioned above, no kinetic data on lactose as the substrate are available.

In this study, we cloned a β-glycosidase gene from the genomic library of *Thermus filiformis* Wai33 A1, and characterized the thermostable β-glycosidase gene and encoded enzyme, especially with respect to lactose hydrolysis under milk pasteurization conditions.

MATERIALS AND METHODS

Bacterial Strains, Vectors, and Media

Thermus filiformis Wai33 A1 (ATCC 43280) cells were cultivated in '*Thermus* Enhanced' medium (ATCC Media Formulations No. 1598) at 70°C in aerobic condition. *E. coli* TOP10F' strain (Invitrogen, U.S.A.) with pUC118 (Takara, Japan) or pGEM-T Easy (Promega, U.S.A.) vector was used for plasmid preparations and gene cloning and *E. coli* BL21(DE3) strain (Novagen, U.K.) with pET21b(+) vector (Novagen, U.K.) was used for expression of His_6-tagged β-glycosidase. All *E. coli* cells with plasmids were grown aerobically in LB (DIFCO, U.S.A.) medium or on LB agar plate at 37°C, supplemented with tetracycline (10 μg/ml) and/or ampicillin (100 μg/ml) for the selection of transformants.

Construction of Genomic Library and Gene Cloning

All the standard recombinant DNA techniques, including plasmid extraction, restriction endonuclease digestion, and DNA ligation, were performed as described in Sambrook *et al.* [24]. Chromosomal DNA of *T. filiformis* Wai33 A1 was extracted by the method of Marmur [15], and gene cloning was carried out according to the procedure of Dion *et al.* [5], with slight modifications. For genomic library construction, chromosomal DNA isolated from *T. filiformis* Wai33 A1 was partially digested with *Sau*3AI, and DNA fragments of 3–9 kb were separated through the sucrose density gradients method with 10–40% (w/v) sucrose solutions [1] and ligated to *Bam*H1-digested pUC118 vector. The ligation products were transformed into *E. coli* Top10F' by electroporation. The transformants were grown to single colonies on the ampicillin and tetracycline containing LB agar plate at 37°C, and the plates were incubated at 70°C for 2 h in order to inactivate the selectable marker, *lacZ*, to prevent ambiguity between *lacZ* and cloned gene. Then, 1.5 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) dissolved in 2 ml Z buffer (0.1 M sodium phosphate, 10 mM KCl, 1 mM $MgSO_4$, 5 mM mercaptoethanol, pH 7.0) [17] were evenly poured onto transformants in the plates. After further incubation for 2 h at 70°C, colonies which turned blue were considered as positive clones to express thermostable enzyme activity. The dead blue colonies were picked up and retransformed into fresh *E. coli* TOP10F' for preparation of clone plasmids. One of the clones containing a 4.5-kb insert, which showed an apparently positive signal to X-gal, was designated as pFI2.

Sequencing and Gene Analysis

The nucleotide sequence of cloned DNA fragment from pFI2 was determined by ABI377 fluorescent sequencing system (Perkin Elmer Applied Biosystems, U.S.A.) with M13 primers and synthetic clone-specific primers. Determination of open reading frame (ORF) and protein alignment were analyzed by PC/GENE software package (V.6.60, IntelliGenetics Inc.). The nucleotide and amino acid sequences were compared with the sequences in the GenBank and SwissProt databases at the National Center for Biotechnology Information (Bethesda, MD, U.S.A.), using the BLAST program.

Overexpression Vector and Enzyme Purification

To construct overexpression vector, PCR amplification was conducted using pFI2 as a template with synthetic primers: TfiglyF (5'TT**CATATG**GCCGAGAACGCCGAAAAGTTT3') and TfiglyR (5'TT**GTCGAC**GAGCTGGGCCCGCGCG'), which introduce *Nde*I and *Sal*I sites on 5'- and 3'-termini of the PCR products, respectively, during amplification (each enzyme site is bold typed). The amplified PCR product was ligated to pGEM-T Easy vector. The TA clone was digested by *Nde*1 and *Sal*1, and ligated to pET21b(+) between the *Nde*1 and *Xho*1 sites.

The resulting plasmid, pEx*tfi*β-*gly*, was transformed into *E. coli* BL21(DE3), and the transformants were aerobically grown to an A_{600} of 1.2 in 150 ml ampicillin-containing LB at 37°C. Then, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM final concentration. After further cultivation for 6 h, the cells were harvested by centrifugation, and the cell pellet was resuspended in 10 ml of 'binding buffer' (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) for Ni^{2+}-affinity-His·binding resin column (Novagen, U.K.). The cell suspension was disrupted by sonication (Sonic Dismembrator 550, Fisher Scientific, U.S.A.) at 200 Hz for 15–20 min, the cell lysate was centrifuged for 20 min at 14,000 ×*g*, and the supernatant was immersed in a 75°C water bath for 30 min. Heat-denatured proteins from *E. coli* were removed by further centrifugation for 20 min at 14,000 ×*g*, and clarified supernatant was then passed through Ni^{2+}-affinity-His·binding resin column (5 ml bed volume), which was charged by 50 mM $NiSO_4$ solution

and then was equilibrated with 15 ml of 'binding buffer.' After loading the sample, the column was washed with 15 ml of 'washing buffer' (Binding buffer containing 60 mM imidazole), and eluted with 15 ml of 'eluting buffer' (Binding buffer containing 1 M imidazole). Among the fractions eluted, the fraction with the highest protein concentration was collected and applied to a Centriplus column (Amicon, U.S.A.) for 120 min at 3,000 ×*g* to desalt and concentrate the enzyme.

Electrophoretic Analysis and Protein Assay

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and nondenaturing electrophoresis (native-PAGE) were carried out, as described in Bollag *et al.* [3], using 4.0% (w/v) acrylamide for stacking gel (pH 6.8) and 7.5% (w/v) for separating gel (pH 8.8). Electrophoresis was performed with a Mini-PROTEAN III system (Bio-Rad, U.S.A.). The protein bands were stained with Coomassie Brilliant Blue R-250 in SDS-PAGE and, for zymogram assay (active staining), the protein bands in native-PAGE were incubated with X-gal in Z buffer (1-1.5 mg/ml) at 70°C. Molecular mass of the purified enzyme was determined indirectly, using the Nondenatured Protein Molecular Weight Marker Kit (Sigma, U.S.A.), as described in the manufacturer's instructions. The proteins used as standard markers were bovine milk α-lactalbumin (14,200 Da), bovine erythrocytes carbonic anhydrase (29,000 Da), chicken egg albumin (45,000 Da), bovine serum albumin (monomer: 66,000 Da; dimer: 132,000 Da), and jack bean urease (272,000: trimer; 545,000: hexamer). Protein concentrations were measured by the Bradford method, using a protein assay kit (Bio-Rad, U.S.A.) with bovine serum albumin as a standard.

Enzyme Assays and Determination of Kinetic Parameters

The specific enzyme activity was measured as the amount of *p*-nitrophenol released from 1 mM *p*-nitrophenyl-β-D-galactoside (pNPGal) or *p*-nitrophenyl-β-D-glucoside (pNPGlc) in 1 ml of Z buffer at 70°C for 15 min using appropriately diluted enzyme preparation. The enzyme reaction was stopped by adding 1 ml of 1 M Na_2CO_3 to the reaction mixture, and absorbance was read at 405 nm using a UV-spectrophotometer (UV-1601 Shimadzu, Japan) and then converted to the amount of *p*-nitrophenol released using a standard curve. Absorbances of blanks were subtracted from all measurements. The enzyme activity on lactose or cellobiose was assayed under the same conditions as described above with the appropriate concentration of substrates. The amount of glucose liberated was measured by the glucose oxidase-peroxidase method using a Glucose C-II kit (Wako, Japan) at 505 nm. One unit of enzyme activity was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol or glucose per minute under the reaction conditions.

The kinetic parameters (K_m and V_{max}) were calculated by Lineweaver-Burk plots using different concentrations of substrates. The ranges of substrate concentration used were 0.01-5 mM for pNPGlc, 1-25 mM for pNPGal, and 10-250 mM for lactose or cellobiose. All the experiments were carried out at 70°C and pH 7.0.

Effect of pH and Temperature on Enzyme Activity

The effect of pH on enzyme activity was determined at 70°C in three buffer systems at 50 mM final concentration: sodium citrate-phosphate buffer from pH 3.0 to 6.0, sodium phosphate buffer from pH 6.0 to 7.5, and borate-Borax buffer from pH 7.5 to 9.0. The effect of temperature on enzyme activity was determined at temperature ranging from 30 to 95°C in Z buffer in a 15-min assay. The thermostability of enzyme was assayed by preincubation of the enzyme at 70, 80, or 90°C in Z buffer, and the

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   1 GATCCTGCCCCTGGCCCTCCCGGGCCTCGTGGCCACGGCCGTCTACATCTTCCTCACCGC  60
  61 CTGGGACGAGCTCCTCTTCGCCCAGGTCCTCACCACCGAGGCCACAGCCACCATTCCCGT 120
 121 GGGCATCCGCAACTTCGTGGGCAACTACCAGAACCGCTACGACCTGGTCATGGCCGCCGC 180
 181 CACGGTGGCCACGCTGCCCGTCCTCGTCGTCTTCTTCTTCGCGCAGCGCCAGCTCATCCA 240
                                          RBS  tfiβ-gly →
 241 AGGCCTCACCGCCGGGGCGGTGAAGGGCTAGGCGTTGGGAGGAGAGAACGCGCATGGCCGA 300
                                                  M  A
 301 GAACGCCGAAAAGTTTCTGTGGGGGGTAGCCACCAGCGCCTACCAGATCGAGGGGGCCAC 360
      E  N  A  E  K  F  L  W  G  V  A  T  S  A  Y  Q  I  E  G  A
 361 CCAGGAGGACGGGCGGGGGCCTTCCATCTGGGACACCTTCGCCCGCCGCCCGGGGGCCAT 420
      T  Q  E  D  G  R  G  P  S  I  W  D  T  F  A  R  R  P  G  A
 421 CCGGGACGGAAGCACAGGGGAGCCCGCCTGCGACCACTACCACCGCTACGAGGAGGACAT 480
      I  R  D  G  S  T  G  E  P  A  C  D  H  Y  H  R  Y  E  E  D
 481 CGCCCTTATGCAATCCCTCGGGGTGGGGGTCTATCGCTTCTCCGTGGCCTGGCCCCGGAT 540
      I  A  L  M  Q  S  L  G  V  G  V  Y  R  F  S  V  A  W  P  R
 541 CCTCCCCGAGGGCCGGGGGCGGATCAACCCCAAGGGCCTCGCCTTTTACGACCGCCTGGT 600
      I  L  P  E  G  R  G  R  I  N  P  K  G  L  A  F  Y  D  R  L
 601 GGACCGGCTTCTCGCGGCGGGGATCACGCCCTTCCTCACCCTCTACCACTGGGACCTGCC 660
      V  D  R  L  L  A  A  G  I  T  P  F  L  T  L  Y  H  W  D  L
 661 CCAGGCCCTCGAGGACCGGGGCGGCTGGCGGAGCCGGGAGACCGCCTTCGCCTTCGCCGA 720
      P  Q  A  L  E  D  R  G  G  W  R  S  R  E  T  A  F  A  F  A
 721 GTACGCCGAGGCGGTGGCCCGGGCCCTCGCCGACCGGGTGCCCTTCTTCGCCACCCTGAA 780
      E  Y  A  E  A  V  A  R  A  L  A  D  R  V  P  F  F  A  T  L
 781 CGAGCCCTGGTGCTCGGCCTTCCTCGGGCACTGGACGGGGGAACACGCCCCCGGCCTCAG 840
      N  E  P  W  C  S  A  F  L  G  H  W  T  G  E  H  A  P  G  L
 841 GAACCTGGAGGCGGCCCTTCGCGCCGCCCACCACCTCCTCCTGGGGCACGGCCTCGCCGT 900
      R  N  L  E  A  A  L  R  A  A  H  H  L  L  L  G  H  G  L  A
 901 GGAGGCCTTGAGGGCCGCGGGGGCGAAGCGGGTGGGGATCGTCCTCAACTTCGCCCCGGT 960
      V  E  A  L  R  A  A  G  A  K  R  V  G  I  V  L  N  F  A  P
 961 GTACGGCGAGGACCCCGAGGCGGTGGACGTGGCTGACCGCTACCACAACCGCTACTTCCT 1020
      V  Y  G  E  D  P  E  A  V  D  V  A  D  R  Y  H  N  R  Y  F
1021 GGACCCCATCCTGGGCAGGGGGTATCCCGAAAGCCCCTTTCAAGACCCCCGCCCACTCC 1080
      L  D  P  I  L  G  R  G  Y  P  E  S  P  F  Q  D  P  P  P  T
1081 CAACCTCTCCCGTGACCTGGAGCTCGTCGCAAGGCCCCTGGACTTCCTAGGAGTGAACTA 1140
      P  N  L  S  R  D  L  E  L  V  A  R  P  L  D  F  L  G  V  N
1141 CTACGCCCCCGTCCGCGTGGCCCCGGGGACGGGGCCTTTGCCCGTGCGCTACCTTCCCCC 1200
      Y  Y  A  P  V  R  V  A  P  G  T  G  P  L  P  V  R  Y  L  P
1201 GGAAGGGCCGGTCACGGCCATGGGGTGGGAGGTCTACCCCGAGGGGCTTTACCACCTCTT 1260
      P  E  G  P  V  T  A  M  G  W  E  V  Y  P  E  G  L  Y  H  L
1261 GAAGCGCCTCGGCCGGGAGGTGCCCTGGCCCCTTTACATCACGGAAAACGGGGCCGCCTA 1320
      L  K  R  L  G  R  E  V  P  W  P  L  Y  I  T  E  N  G  A  A
1321 CCCCGACCTCTGGACGGGAGAGGCCGTCGTGGAGGACCCCGAGCGGGTGGCCTACCTCGA 1380
      Y  P  D  L  W  T  G  E  A  V  V  E  D  P  E  R  V  A  Y  L
1381 GGCCCACGTGGAGGCCGCCCTCCGGGCCCGGGAGGAAGGGGTGGACCTCAGGGGCTACTT 1440
      E  A  H  V  E  A  A  L  R  A  R  E  E  G  V  D  L  R  G  Y
1441 CGTCTGGAGCCTCATGGACAACTTTGAGTGGGCCTTCGGCTACACCCGGCGCTTCGGCCT 1500
      F  V  W  S  L  M  D  N  F  E  W  A  F  G  Y  T  R  R  F  G
1501 CTACTACGTGGACTTCCCCAGCCAGAGGCGCATCCCTAAAAGGAGCGCCCTCTGGTACCG 1560
      L  Y  Y  V  D  F  P  S  Q  R  R  I  P  K  R  S  A  L  W  Y
1561 GGAGCGGATCGCGCGGGCCCAGCTCTGAGCGTCCGCGCATTGATCCCGCCACTTAGGGCC 1620
      R  E  R  I  A  R  A  Q  L  -
1621 CCCGAACCGGGGAGGGTGGACGGCGAGGACTCCAGAGGCCTCTACCCTCCCCATAAGGGC 1680
1681 CTCGAGGTCCCGGGAAGAGGCCCTAAAACTCAAAGAAGGCCGGGACGAAGGCGTAGCC   1738
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Fig. 1. Nucleotide and deduced amino acid sequences of the *tfi* β-*gly* gene (GenBank accession number AY130256).
RBS, the presumed ribosome-binding site, is indicated in the dark box, and the two conserved amino acid motifs of glycosyl hydrolase family 1 are underlined.

residual activity was measured at 70°C with withdrawn aliquots at intervals. All assays were performed using pNPGal as a substrate under the standard conditions.

RESULTS

Cloning and Sequencing of the β-Glycosidase Gene from *T. filiformis* Wai33 A1

Approximately 6,000 recombinant *E. coli* Top10F' colonies were generated from the genomic library of *T. filiformis* Wai33 A1, and two clones were found to be positive to X-gal at 70°C. These clones contained 4.5 and 5.6 kb inserts, respectively. Because of similar patterns of restriction enzyme mapping, we predicted that the inserts of two clones were originated from the same region of *T. filiformis* Wai33 A1 DNA (data not shown). Thus, we selected the recombinant plasmid harboring the 4.5-kb insert, the shorter one, and it was designated as pFI2.

To analyze the structure of the gene encoding β-glycosidase enzyme, the insert of pFI2 was sequenced by primer walking using M13 and synthetic primers (Fig. 1). 1,738 bp of the 4.5 kb insert were determined from the positive direction to the *lac* promoter of the pUC118 vector. Its overall G+C content was 69.7%, and the BLAST program detected a 1,296 bp-ORF from the sequenced part of the insert. The ORF encoded a polypeptide of 431 amino acids with deduced molecular mass of 48,630 Da. It was highly related to other published β-glycosidases, belonging to the glycosyl hydrolase family 1, and two known catalytic motifs (Asn-Glu-Pro and Glu-Asn-Gly) for glycosyl hydrolase were detected in the ORF [5, 9, 10, 18]. Thus, we designated the ORF as *tfi β-gly*. No promoter-like sequence, which can function in *E. coli*, was found in the upstream region of *tfi β-gly*; however, a putative ribosome binding site (RBS) was detected upstream of the translation start codon of the gene.

The amino acid sequence deduced from *tfi β-gly* was compared with other known β-glycosidases. This analysis revealed that the amino acid sequence encoded by *tfi β-gly* scored a high identity with others from *Thermus* species (Fig. 2): 99% with *T. caldophilus* GK24 [9], 97% with *T.*

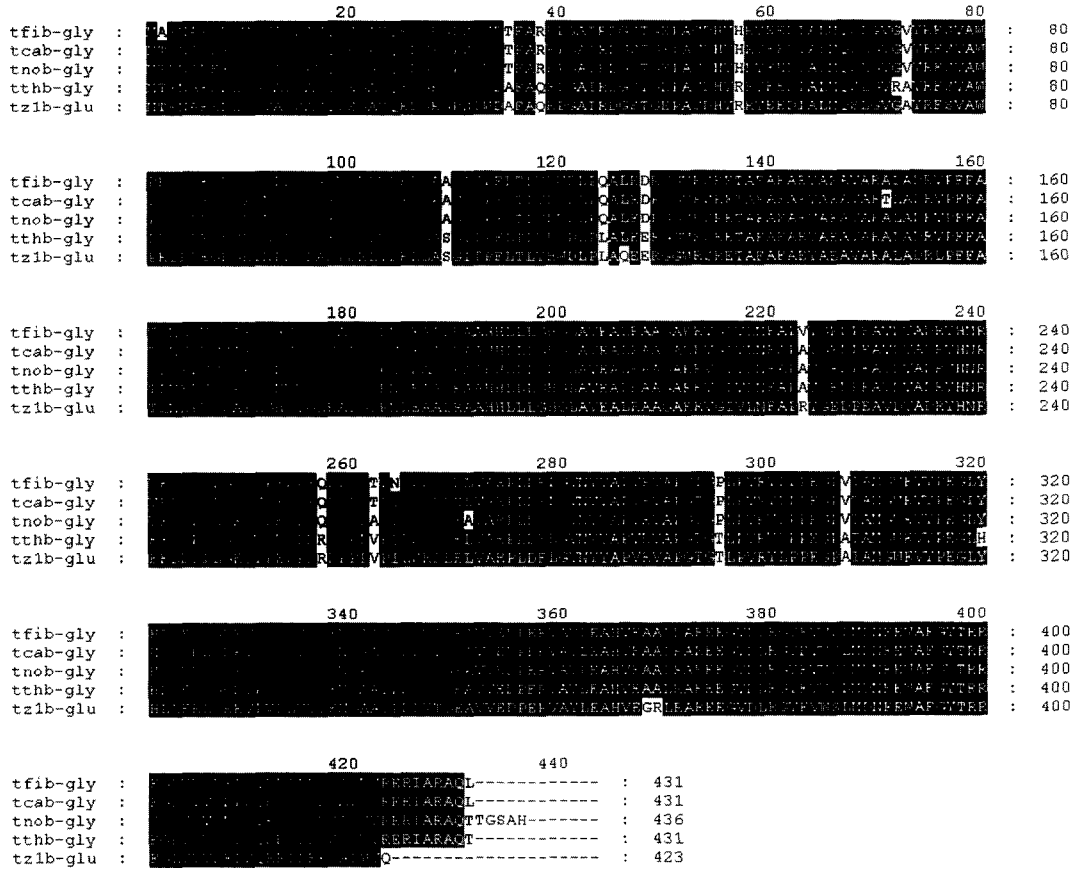

Fig. 2. Amino acid sequence alignment of Tfi β-glycosidase with other β-glycosidases from *Thermus* spp.
Abbreviations and GenBank accession numbers: tfib-gly, *T. filiformis* Wai33 A1 (this study); tcab-gly, *T. caldophilus* GK24 (AF322356); tnoβ-gly, *T. nonproteolyticus* HG102 (AF225213); tthβ-gly, *T. thermophilus* HB27 (Y16753); tz1b-glu, *Thermus* sp. Z1 (BAA86923). Dark and light shadings show identical and conserved residues, respectively, among the sequences.

Table 1. Purification procedure for extracting Tfi β-glycosidase.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude extract	59.4	509.8	8.6	1.0	100
Heat precipitation	13.5	471.2	34.9	4.1	92.4
His·binding column[a]	4.9	368.5	75.2	8.7	72.3

[a]The concentration of protein and enzyme activity were measured after desalting by Centriplus column.

nonproteoliticus HG102 [10], 95% with *T. thermophilus* HB27 [5], and 93% with *T.* sp. Z-1 [26], thus having the highest identity with *T. caldophilus* GK24. There were only 4 amino acid residues different in the sequences between Tfi β-glycosidase and *T. caldophilus* GK24. *T. nonproteoliticus* HG102 was also highly identical to that of Tfi β-glycosidase, except its C-terminal sequences: *T. nonproteoliticus* HG102 had 5 additional amino acid residues at its C-terminus.

Overexpression and Purification of Tfi β-Glycosidase

For the production of the recombinant Tfi β-glycosidase enzyme, the transformant harboring pEx*tfi*β-*gly* was cultivated in 150 ml of LB/Amp medium with 1 mM of IPTG at 37°C. The His_6-tagged β-glycosidase enzyme was mainly expressed as a soluble protein in the cytosol of *E. coli* cells.

Tfi β-glycosidase was purified by the method described in Materials and Methods. Heat treatment at 75°C greatly facilitated further purification, which required just a single chromatographic step using a Ni^{2+}-affinity-His·binding column to obtain homogenous form of the enzyme. The enzyme was purified to 8.7-fold with 72.3% yield (Table 1), and a single band between the 45 and 66 kDa regions was detected by SDS-PAGE (Fig. 3A). A zymogram assay performed on native-PAGE with X-gal as a substrate at 70°C revealed that the purified protein corresponded to the Tfi β-glycosidase enzyme (Fig. 3B).

We also determined the precise molecular mass of the Tfi β-glycosidase using the Nondenatured Protein Molecular Weight Marker Kit (Sigma). Thus, the purified enzyme preparation was compared with standard marker proteins on a set of various concentrations of nondenatured polyacrylamide gel, and the estimated molecular mass of Tfi β-glycosidase was approximately 49 kDa (data not shown). It was well consistent with the molecular mass deduced from the amino acid sequence and with the result of SDS-PAGE, indicating that Tfi β-glycosidase is a monomeric enzyme.

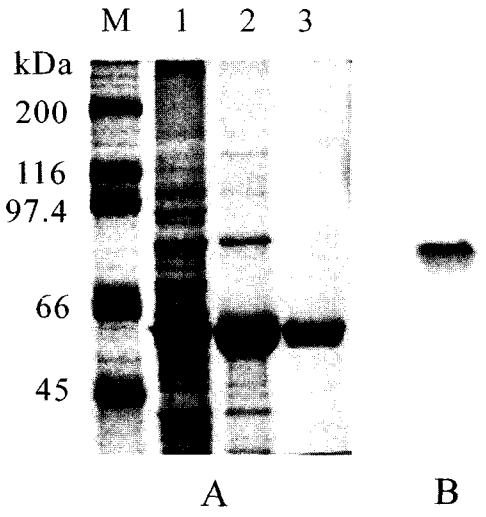

Fig. 3. SDS-PAGE and zymogram analysis of Tfi β-glycosidase.
The gels were stained with Coomassie Brilliant Blue in SDS-PAGE and with X-gal in the zymogram under nondenatured condition, respectively. (A) Lane M, Broad Range, the molecular standard marker (Bio-Rad, U.S.A.); lane 1, cellular extract of pEx*tfi*β-*gly*-transformed *E. coli* BL21(DE3); lane 2, clarified cellular extract of transformants after heat treatment at 75°C for 30 min; lane 3, purified Tfi β-glycosidase from the Ni^{2+}-affinity-His·binding column chromatography. (B) Purified Tfi β-glycosidase stained with X-gal at 70°C for 15 min after native-PAGE.

Optimal pH, Optimal Temperature, and Thermostability of the Enzyme

To determine the optimal pH of the enzyme, the activity of Tfi β-glycosidase was examined at 70°C and pH range of 3.0 and 9.0 with pNPGal as a substrate. A maximum activity was detected at pH 5.0–6.0 (Fig. 4). The enzyme retained more than 80% of the activity in the pH range of 4.5–6.0. Enzyme activity was also assayed at temperatures

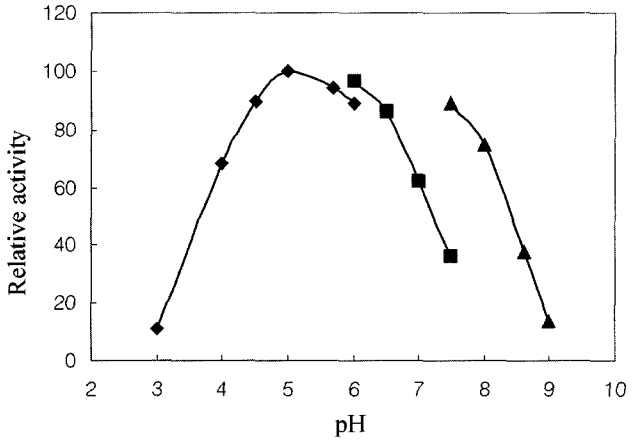

Fig. 4. Effect of pH on the enzyme activity.
The assay was carried out at 70°C with pNPGal as a substrate, using sodium citrate-phosphate buffer (◆), sodium phosphate buffer (■), and borate-Borax buffer (▲).

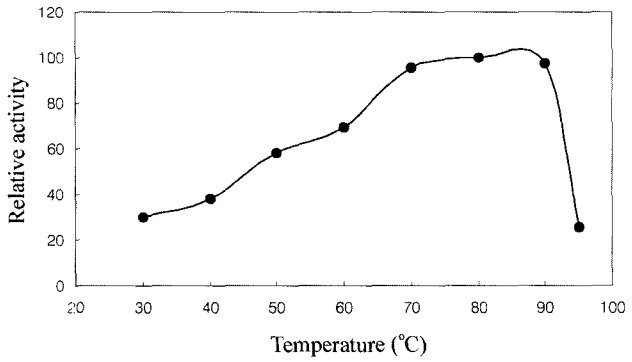

Fig. 5. Effect of temperature on the enzyme activity.
Tfi β-glycosidase activity was assayed at temperature range from 30 to 95°C and pH 7.0 with pNPGal as a substrate.

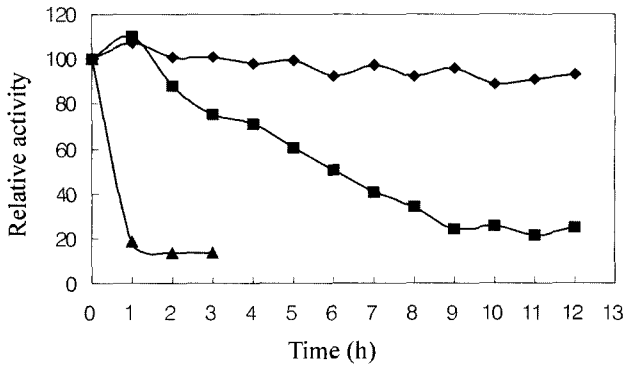

Fig. 6. Thermostability profile of the enzyme.
The purified enzyme was preincubated at 70 (◆), 80 (■), and 90°C (▲) for various time periods, and the residual activity was measured with pNPGal as a substrate at 70°C and pH 7.0.

ranging from 30 to 95°C at pH 7.0. As shown in Fig. 5, the enzyme activity increased as the temperature was raised and showed its maximum activity at around 80-90°C, then sharply decreased at above 90°C. Tfi β-glycosidase was highly thermostable, so that the enzyme retained 93% of its activity after 12-h of incubation at 70°C (Fig. 6). The half-life of the enzyme at 80°C was 6 h.

Kinetic Properties of the Enzyme

To observe the kinetic behaviors, the enzyme activity was assayed by monitoring the hydrolysis of β-D-glycosides at various concentrations, and the kinetic parameters of β-glycosidases from *Thermus* spp. are summarized in Table 2. The K_{cat}/K_m values of Tfi β-glycosidase for pNPGlc and pNPGal were 823.6 and 68.3 respectively, indicating that the enzyme is more specific to pNPGlc. However, the K_{cat} value for pNPGal was 2.5 times higher than that of pNPGlc.

We also examined the hydrolysis of lactose and cellobiose at 70°C and pH 7.0. The K_{cat} and K_m values of Tfi β-glycosidase for cellobiose were 97 sec^{-1} and 43 mM, respectively. However, the plot of reaction velocity (v: μmol/mg/min) versus substrate concentration ([s]) for lactose did not show standard Michaelis-Menten kinetics (Fig. 7). The reaction velocity of the enzyme for lactose hydrolysis increased continuously without substrate inhibition until the lactose concentration reached 250 mM. At low substrate concentrations (<90 mM), the reaction velocity with cellobiose was slightly higher than that of lactose. However, the activity with lactose was much higher than that of cellobiose, when the substrate concentration was raised to above 90 mM. This indicates that Tfi β-glycosidase might be more effective for lactose hydrolysis than for cellobiose hydrolysis at high substrate concentration.

Normal bovine milk contains an average 4.7% lactose (approximately 138 mM) [28]. The specific activity of Tfi β-glycosidase on 138 mM lactose at 70°C and pH 7.0 was 134.9 U/mg (Fig. 7).

Table 2. Kinetic parameters of β-glycosidases from *Thermus* spp.

Strain	Substrate	Specific activity U/mg (substrate)	K_{cat} (K_m)	Assays at		Ref.
				pH	Temp. (°C)	
T. filiformis Wai 33 A1	pNPGal	75.2 (1 mM)	628.4 (9.2)	7.0	70	This study
	pNPGlc		255.3 (0.31)	7.0	70	
	lactose	134.9 (138 mM)	ND[a]	7.0	70	
	cellobiose		97 (43)	7.0	70	
T. thermophilus HB27	pNPGal		118 (6.6)	7.0	65	5
	pNPGlc		23 (0.09)	7.0	65	
	cellobiose		41 (4.0)	7.0	70	
T. caldophilus GK24	pNPGal	83 (5 mM)		6.0	75	9
T. nonproteolyticus HG102	pNPGal		83 (10)	6.0	80	10
	pNPGlc		92 (0.9)	6.0	80	
	lactose	transglycosyl activity		6.0	65	

[a]ND, not determined. Enzyme activity increased linearly with increase of substrate concentration in 250 mM range of lactose. At 250 mM, the K_{cat} (sec^{-1}) value was 177.

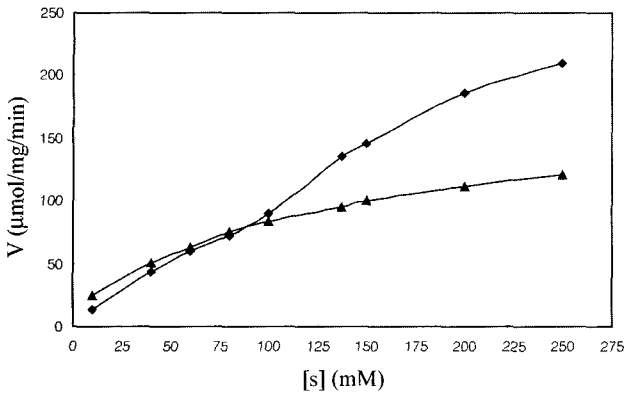

Fig. 7. Effect of lactose (◆) and cellobiose (▲) concentrations on the velocity of the enzyme-catalyzed reaction.
The specific enzyme activitiy was determined using different concentrations of lactose or cellobiose, ranging from 10 to 250 mM at 70°C and pH 7.0.

DISCUSSION

A gene encoding thermostable β-glycosidase from the genomic library of *Thermus filiformis* Wai33 A1 was cloned, sequenced, and expressed in *E. coli*. The sequence of the cloned gene, *tfi* β-*gly*, consisted of 1,296 nucleotides and encoded a polypeptide of 431 amino acids. It displayed strong homology with other β-glycosidases from *Thermus* sp. which belong to glycosyl hydrolase family 1. However, in spite of high similarity in their N-terminal and internal residues, *Thermus* β-glycosidases had different lengths of amino acid sequence.

Although *tfi* β-*gly* successfully expressed corresponding enzyme in *E. coli*, no typical promoter-like sequence was found in the upstream region of cloned DNA. pFI2 and the other clone showing positive signal to X-gal had inserts correctly oriented to the *lac* promoter of the cloning vector. Thus, in the *E. coli* system, the expression of *tfi* β-*gly* appears to be controlled by the *lac* promoter rather than by its own promoter, because this seems to be a general tendency of *Thermus* spp.-derived cloned genes in *E. coli* due to difference between the two bacteria in recognition signals for RNA polymerase [5, 14, 16].

The expression vector, pEx*tfi*β-*gly*, highly expressed His_6-tagged Tfi β-glycosidase as a soluble form in the cytosol of host *E. coli* cells. The cellular enzyme activity was measured at various stages of cell growth and at a series of induction time to determine optimum induction condition, and the best induction condition was found to be 6 h-incubation after IPTG treatment at A_{600} of 1.2 (data not shown). Heat precipitation was quite effective, since most heat-labile proteins from the host cells could be removed and the recombinant enzyme was obtained as a homogenous form simply through a single chromatography with the Ni^{2+}-affinity-His-binding column. Tfi β-glycosidase was a monomeric enzyme of 49 kDa, and it was consistent with other β-glycosidases from *Thermus* spp. [5, 9, 10].

The optimal pH and temperature of Tfi β-glycosidase was pH 5.0-6.0 and 80-90°C. This pH range represents a usual phenomenon, since most β-glycosidases from bacterial sources show their pH optima in slightly acidic or neutral pH ranges [8]. One of the generally used methods for milk pasteurization is the LTLT (Low-Temperature, Long-Time) process, which involves heating of milk to a temperature between 62.8°C and 65.6°C for 30 min [24]. Regarding pH and temperature optima of the enzyme, Tfi β-glycosidase is potentially well suited for the enzyme application in low-lactose milk production during the LTLT process. Tfi β-glycosidase retained more than 80% of its activity at pH range of 4.5-6.5, corresponding to pH 6.7 of normal bovine milk, and displayed 70-90% of its activity at temperature range of 60-70°C. Moreover, Tfi β-glycosidase was highly thermostable, so that it retained 93% of its activity after 12 h at 70°C. This thermostability of the enzyme allows higher operating temperature and increases the half-life of the enzyme, consquently resulting in preventing economic losses caused by frequent exchange of enzyme in the industrial application.

Based on K_m and K_{cat}/K_m, Tfi β-glycosidase showed much higher substrate-specificity on pNPGlc than on pNPGal. However, the K_{cat} value for pNPGal was 2.5 times higher than that for pNPGlc (Table 2), suggesting that the enzyme could be more easily saturated by β-D-glucoside than by β-D-galactoside even at very low concentration of the substrate, because of its high affinity for β-D-glucoside. This characteristic might lower the maximum capacity of the enzyme in the hydrolysis of pNPGlc at high substrate concentration. Similar results were also obtained in the case of β-glycosidase from *T. thermophilus* and *T. nonproteolyticus* [5, 10]. However, detailed kinetic values for each enzyme were widely different, although β-glycosidases from *Thermus* spp. shared over 93% sequence identity with each other (Fig. 2, Table 2). For example, the kinetic values for cellobiose were quite different between Tfi β-glycosidase and Tt β-gly from *Thermus thermophilus* HB27 though they shared 95% sequence identity with each other. Even though Tt β-gly has an over 10 times lower K_m value for cellobiose than that of Tfi β-glycosidase, the K_{cat} value with Tfi β-glycosidase was 2.4 times higher than with Tt β-gly. It is difficult to compare enzyme to enzyme directly, because of different assay conditions for each enzyme; nevertheless, Tfi β-glycosidase showed relatively much higher K_{cat} values for pNPGal, pNPGlc, or cellobiose than β-glycosidases from other *Thermus* spp., independent of their K_m values (Table 2).

Three β-glycosidases so far have been reported in *Thermus* spp. [5, 9, 10], but no kinetic data on lactose are available. Thus, we assayed lactose hydrolysis of Tfi β-

glycosidase at pH 7.0 and 70°C (similar to the LTLT condition) in order to test whether it would be suitable for application in low lactose-milk production during milk pasteurization. Very interestingly, the reaction velocity of the enzyme for lactose showed a linear increase without substrate inhibition until lactose concentration up to 250 mM was examined (Fig. 7). Such activation effect at high substrate concentrations on various β-D-glycosides has already been observed with many β-glycosidases [2, 4, 7, 13, 19, 21]. It was also reported that the activation phenomenon at high substrate concentration was due to the transgalactosylation activity of β-glycosidase [7]. However, more detailed study is needed to confirm whether Tfi β-glycosidase on lactose falls into this category. The activation effect of the enzyme at high concentration of substrate and high temperature could facilitate stable enzymatic reaction during milk pasteurization in batch type methods such as the LTLT process. The specific activity of Tfi β-glycosidase on 138 mM lactose (normal lactose concentration of bovine milk) was 134.9 U/mg at 70°C and pH 7.0 (Fig. 7). Consequently, 1 g of Tfi β-glycosidase has the capability to hydrolyze total amount of lactose present in 1-l of bovine milk within about 1 min during milk pasteurization. Moreover, its specific activity was more than 10 times higher than that of β-galactosidase from *Thermus* sp. T2 on 138 mM lactose (13 U/mg) under the same condition [27]. This suggests that, among the lactose hydrolases from *Thermus* spp., β-glycosidase is more suited for lactose hydrolysis than β-galactosidase under milk pasteurization conditions.

In conclusion, considering the optimal pH and temperature, thermostability, and hydrolysis of lactose, Tfi β-glycosidase is potentially very much suited for the use in low-lactose milk production during milk pasteurization. Direct application of Tfi β-glycosidase toward this approach is now in progress in our laboratory.

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