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Cloning and Expression of Thermostable β-Glycosidase Gene from *Thermus nonproteolyticus* HG102 and Characterization of Recombinant Enzyme

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Abstract

The gene coding for β-glycosidase (EC 3.2.1.21) from *Thermus nonproteolyticus* HG102 was cloned and expressed in *Escherichia coli*. The gene open reading frame was 1311 bp, and it codes for 437 amino acids. The deduced amino acid sequence of the enzyme showed identity with members of the glycosyl hydrolase family I. The enzyme had high content of Arg and Pro. The recombinant enzyme was purified to homogeneity with heat precipitation, ammonium sulfate precipitation, DEAE-cellulose (DE52) chromatography, and prepared slab polyacrylamide gel electrophoresis. The enzyme was monomeric and had a molecular mass of 48,900 Daltons and a p*I* of 5.2. The enzyme showed optimum activity at pH 5.6 and 90°C. It catalyzed the hydrolysis of β-D-glucoside, β-D-galactoside, β-D-fucoside, and β-D-mannoside. Lineweaver-Burk plots showed that the k_{cat}/K_m ratio for β-D-glucoside and β-D-fucoside was higher than for β-D-mannoside and β-D-galactoside. The enzyme was extremely thermostable, with a half-life of 2.5 h at 90°C, and was stable over a wide range of pH. It also had transglycosidic activity at high temperature.

Index Entries: *Thermus nonproteolyticus* HG102; thermostable β-glycosidase; gene cloning and expression.

Introduction

β-Glycosidases (EC 3.2.1.21) are enzymes that catalyze the hydrolytic cleavage of β-glycosyl linkages. They are ubiquitous enzymes that have been isolated and characterized from various organisms. These enzymes

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are characterized by a broad substrate specificity that make them potential tools for several applications *(1)*. In this regard, β-glycosidases from thermophilic sources are particularly attractive because of their biotechnologic advantages for any stabilized biocatalysts. Furthermore, they may contribute to the study of the structure-function relationship of thermophilic enzymes by comparing their properties with those from mesophilic enzymes *(2)*. Several thermostable β-glycosidases have been purified or cloned: *Pyrococcus furiosus (3)*, *Sulfolobus solfataricus (4)*, *Thermotoga maritima (5)*, *Thermosphaera aggregans (6)*, and *Thermus thermophilus (7)*.

Thermus nonproteolyticus HG102 was isolated from a hot spring in Guangdong Province, southern China. It grows optimally at 65°C *(8)*. In this article, we report gene cloning, expression, and characterization of β-glycosidase from *T. nonproteolyticus* HG102.

Materials and Methods

Materials

Restriction enzymes and T4 ligase were purchased from Boehringer Mannheim. Protein molecular markers and the p*I* protein kit were from Pharmacia. DEAE-cellulose (DE52) was obtained from Whatman. *p*-Nitrophenyl-β-D-glucopyranoside, *p*-nitrophenyl-β-D-galactopyranoside, *p*-nitrophenyl-β-D-fucopyranoside, *p*-nitrophenyl-β-D-mannopyranoside, lactose, and cellobiose were purchased from Sigma. Other chemicals used were of analytical grade.

Bacterial Strains, Plasmids, and Media

T. nonproteolyticus HG102 was cultivated at 65°C in medium containing 0.8% (w/v%) peptone, 0.5% (w/v%) yeast extract, 0.2% (w/v%) NaCl, 0.5% (w/v%) lactose, and 0.05% (w/v%) glucose. *Escherichia coli* AS 1.1739 [K12r$^-$Δ (lacIPOZY) × 74] was obtained from the Institute of Microbiology, Chinese Academy of Sciences. The culture medium was Luria-Bertani (LB) broth and cultivated at 37°C. The selection medium was ampicillin-resistant (100 μg/mL) LB broth supplied with isopropylthio-β-D-galactoside (IPTG) and X-gal as in Sambrook et al. *(9)*. The clone plasmid was pUC18.

DNA Manipulation and Cloning of β-Glycosidase

Chromosomal DNA was extracted from *T. nonproteolyticus* HG102 according to the method of Marmur *(10)*. Plasmid extraction and restriction endonuclease digestion and other standard recombinant DNA techniques were done as described in Sambrook et al. *(9)*.

Chromosomal DNA from *T. nonproteolyticus* HG102 was partially digested with *Hin*dIII and then ligated into *Hin*dIII-digested and dephosphorylated pUC18 vector using T4 DNA ligase. The ligation mixture was then used to transform *E. coli* AS1.1739. Recombinant *E. coli* strains were cultivated at 37°C and grown to single colonies on the selection medium. The

plates were incubated at 50°C for 2–5 h. The blue colonies, which were positive colonies, were chosen to be cultivated on Amp-LB at 37°C for further testing. The recombinant plasmid was designated as pHY.

DNA Sequencing and Analysis

The sequence of the inserted DNA was determined on an AB1377 fluorescent sequencing system using the pHY plasmid as template. The sequence was compared with Genbank and Swissprot databanks using the Gapped BLAST program *(11)*. The alignments and secondary structure predictions were performed by means of the DNASTAR Windows 32 program.

Purification of Recombinant Protein

The pHY was used to transform *E. coli* AS1.1739. Transformants were cultivated in 2 L of LB medium containing ampicillin (100 μg/mL) and 1% lactose (w/v%) at 37°C for 24 h. The cells were harvested by centrifugation, resuspended in 50 m*M* phosphate buffer (pH 6.6), and disrupted by sonification. After centrifugation (12,000g, 30 min), the soluble cell-free extract was heat precipitated for 15 min at 80°C. Ammonium sulfate cuts between 30 and 60% final saturation were performed. The precipitate was then dissolved in 50 m*M* phosphate buffer (pH 6.6). The supernatant was applied to a DEAE-cellulose (DE52) column and eluted from the column with a linear NaCl gradient (0.05–0.15 *M*) in the same buffer. The enzyme was pooled and further purified by preparative native slab polyacrylamide gel electrophoresis (PAGE). After electrophoresis, the gels were stained for activity with 4 m*M* pNPGlucopyranoside at 80°C. The enzyme band was cut out of the gel and dissolved in 50 m*M* phosphate buffer (pH 6.6) overnight, and the protein was then concentrated (12,000*g*, 30 min). The supernatant was dialyzed against the phosphate buffer. The purified enzyme obtained by these procedures was used for analysis of the enzymatic characteristics.

*Determination of Molecular Mass and p*I

The molecular mass of the enzyme was determined by gel filtration chromatography and sodium dodecyl sulfate (SDS)-PAGE. The gel filtration was a Superdex G-75 column on FPLC (Pharmacia) equilibrated with 20 m*M* phosphate buffer (pH 7.0) containing 0.15 *M* NaCl. SDS-PAGE in slab gels was performed according to the method of Laemmli *(12)*. The standard proteins used were phosphorylase b (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa). Electrophoresis was performed at a constant voltage of 150 V for 1.5 h. The gels were stained with Coomassie brilliant blue R-250.

The p*I* of the purified enzyme was determined according to the procedure suggested by Vesterberg *(13)*.

Enzymatic Reactions with Oligosaccharides

The transglycosylation activity of the recombinant enzyme was tested at 65°C using lactose and cellobiose as substrates. The reaction mixture was 50 m*M* sodium phosphate buffer (pH 6.0) with 5 m*M* substrate and 5.0 µg/mL of enzyme. The products were analyzed using thin-layer chromatography (TLC) of silica gel 60 (Merck) using isopropanol:acetone:H_2O (2:2:1 [v/v/v]) as solute. The plate was stained with aniline-diphenylamine color-developing solution at 85°C for 10 min.

Enzyme Assay, Kinetic Parameters, and Determination of Protein

Protein concentrations were determined using the method of Lowry et al. *(14)* with bovine serum albumin as the standard. The enzyme activity was measured at 80°C. The reaction solution containing 0.7 mL of H_2O, 0.1 mL of 50 m*M* phosphate buffer (pH 6.0), and 0.1 mL of 4 m*M* pNP-glycopyranoside was prewarmed for 5 min, and 0.1 mL of enzyme sample was then added to the reaction mixture for 10 min and stopped by adding 4 mL of 0.4 *M* Na_2CO_3. The released *p*-nitrophenol was measured at 420 nm. The reference cell contained all reactants except the enzyme. One unit was defined as the amount of enzyme that cleaved 1 µmol of substrate/min.

The kinetic parameters (K_m and k_{cat}) were determined using Lineweaver-Burk *(15)* plots with different concentrations of *p*-nitrophenyl-β-D-glycopyranoside (0.05–10 m*M*). Each experimental point was determined at least three times, and in all cases the initial rate was used for plotting.

Effect of pH and Temperature on Activity and Stability

The effect of pH on the enzyme activity was determined by measuring the hydrolysis of pNPGlucopyranoside in a series of buffers at various pHs. The buffers were 100 m*M* sodium acetate buffer (pH 3.6–5.8), 100 m*M* phosphate buffer (pH 5.8–7.8), 100 m*M* Tris-HCl (pH 7.8–8.8), and 100 m*M* glycine-NaOH (pH 8.8–10.0). The pH values of each buffer were determined at 25°C. The experiments studied the dependence of the activity on temperature at temperatures ranging from 35 to 95°C in phosphate buffer (50 m*M*, pH 6.0) for 10 min to assay the activity. The thermostability of the enzyme was studied at 70, 80, and 90°C by heating the enzyme solutions in phosphate buffer (50 m*M*, pH 6.0) and removing aliquots at different times to measure the residual activity using the standard method. The pH stability was studied by preincubating the enzyme solutions in different pH buffers for 30 min at 50°C and then measuring the residual activity using the standard method.

Results

Cloning of β-Glycosidase Gene

Of about 10,000 transformants derived from the *Hin*dIII library, three positive matches were found that produced thermostable β-glycosidase. The recombinant plasmids (pHY) isolated from these three transformants

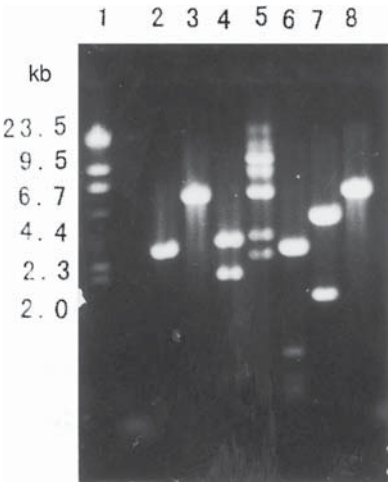

Fig. 1. Restriction enzyme map of the fragments cloned in plasmid pHY. Lane 1, λDNA digested by *Hin*dIII; lane 2, pHY digested by *Hin*dIII; lane 3, pHY digested by *Eco*RI; lane 4, pHY digested by *Bam*HI; lane 5, pHY digested by *Sac*I; lane 6, pHY digested by *Sma*I; lane 7, pHY digested by *Kpn*I; lane 8, pPHY digested by *Pst*I.

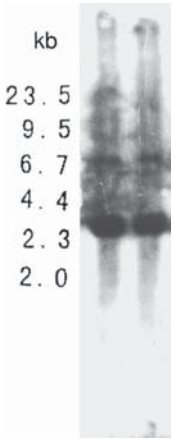

Fig. 2. Southern hybridization analysis using the cloned plasmids as probes against *Thermus n.* HG102 genomic DNA. The two lanes are both genomic DNA digested by *Hin*dIII, and the numbers on the left indicate kilobase scale (λDNA fragments digested by *Hin*dIII).

all had an inserted DNA fragment of about 2.6 kb. Restriction enzyme analysis of this recombinant plasmid pHY is shown in Fig. 1. It shows the restriction sites for *Bam*HI, *Sac*I, *Sma*I, and *Kpn*I. Southern hybridization indicated that the inserted fragment was homologous with *T. nonproteolyticus* HG102 genomic DNA (Fig. 2). The *E. coli* AS1.1739 with recombinant plasmid pHY can produce thermostable β-glycosidase (Fig. 3). The specific activity of the recombinant enzyme (115 U/mg) was 17-fold greater than the native enzyme (6.7 U/mg) produced by *T. nonproteolyticus* HG102.

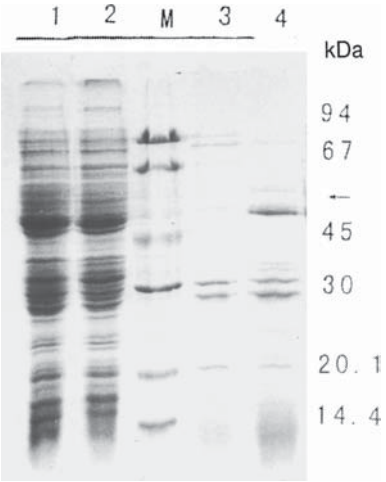

Fig. 3. SDS-PAGE of *T.n.-Gly* expression in *E. coli* AS1.1739. Lane 1, *E. coli* AS1.1739 (pUC18) cell-free extract; lane 2, *E. coli* AS1.1739 (pHY) cell-free extract; lane 3, *E. coli* AS1.1739 (pUC18) cell-free extract heated at 80°C for 15 min; lane 4, *E. coli* AS1.1739 (pHY) cell-free extract heated at 80°C for 15 min. M, standard marker proteins.

Sequencing and Analysis β*-Glycosidase Gene*

The entire sequence of the inserted DNA was determined and the length was found to be 2623 bp. The BLAST program revealed the presence of the 1311-bp open reading frame (ORF) displaying strong homology with other reported β-glycosidase genes (data not shown), which suggests that the 1311-bp ORF was the *T.n.* β-glycosidase gene. The *T.n.* β-glycosidase gene (GeneBank accession no. AF225213) and the flanked sequence are presented in Fig. 4. The gene starts at 189 (ATG) and continues to 1499 (TGA) and coded for a protein of 437 amino acids with a predicted molecular mass of 48,997 Daltons. The G+C content was 71%. The possible ribosome-binding site (RBS) was located 8 bp upstream of the translation start (ATG), but typical promoter sequence was not found. The amino acid composition of the enzyme had high Arg (9.6%) and Pro (8.0%) content, and a high Arg/Lys ratio (10.5).

The amino acid sequence deduced from the encoded *T.n.* β-glycosidase gene was compared with other glycosyl hydrolases whose sequences are available in databases. This analysis revealed that the enzyme is a member of the glycosyl hydrolase family I. Other β-glycosidases having identities with *T.n.* β-glycosidase higher than 40% were *Streptomyces* sp. β-glu (46%) *(16)*, *Thermoanaerobacter brockii* β-glu (46%) *(17)*, *T. maritima* β-glu (44%) *(18)*, *Caldocellum saccharolyticum* β-glu (43%) *(19)*, *Bacillus circulans* β-glu (44%) *(20)*, *Microbispora bispora* β-glu (46%) *(21)*, *Bacillus polymyxa* β-glu (42%) *(22)*, and *Thermotoga neapolitana* β-glu (43%) *(23)*. The identity with Archea β-glycosidase *(3,4)* was much lower (about 28%). The identity of the amino acid sequences between *T. nonproteolyticus* HG102

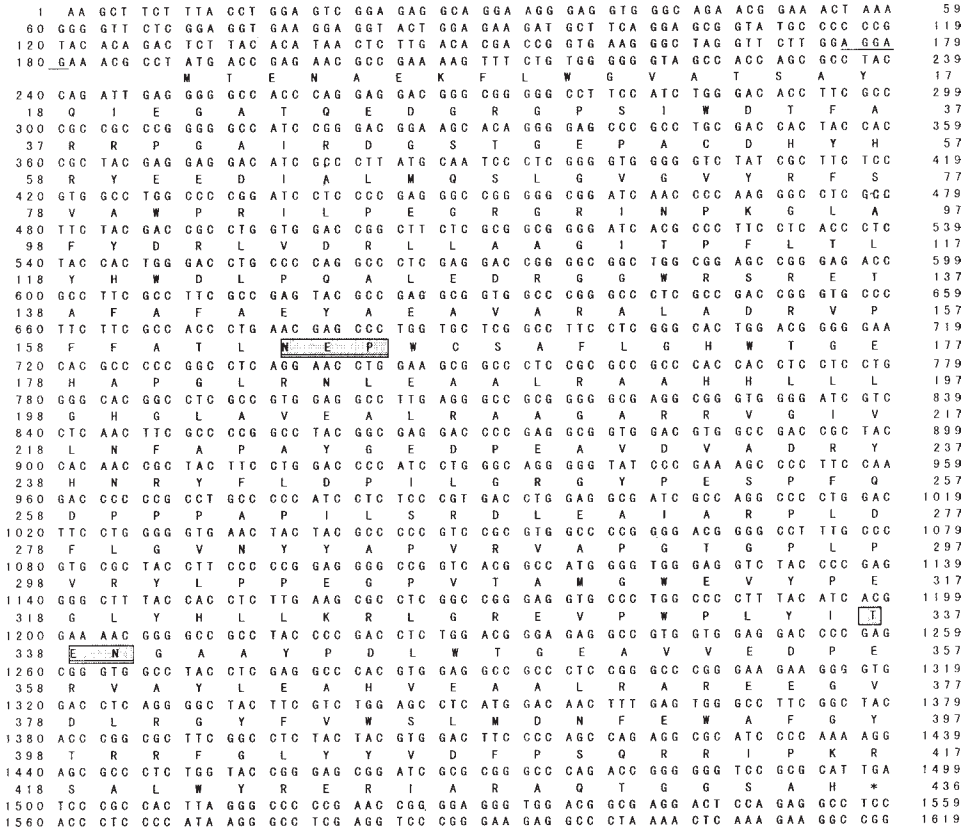

Fig. 4. Nucleotide and deduced amino acid sequences of *T.n.-Gly* (Genebank accession no. AF225213). The putative RBS is underlined, the conserved amino acid is boxed, and asterisk represents the stop codon.

and *T. thermophilus (7)* was 85%, with the differences primarily within the N- and C-terminal sequences. This difference did not affect the enzymatic character. Comparison of the alignments of the *T.n.* β-glycosidase amino acid sequence with different sequences of glycosyl hydrolase family I (Fig. 5) showed that *T.n.* β-glycosidase has the conserved catalytic domains -N-E-P- and -T-E-N-, which are fully conserved among glycosyl hydrolases from mesophilic and thermophilic bacteria as described by Moracci et al. *(24)*.

The Protean program of DNASTAR was used to predict the secondary structure. According to the Chou-Fasman *(25)* model, the enzyme has 41.4% α-helices, 16.2% β-strands, and 14.4% β-turns, and 14 of the 35 Pro were located at the second sites of β-turns.

Purification of Recombinant Protein

Table 1 summarizes the results of the purification of recombinant thermostable β-glycosidase. The enzyme was purified 36.9-fold with 9.5% activity recovery. Heat treatment of the cell-free extract was an effective purification

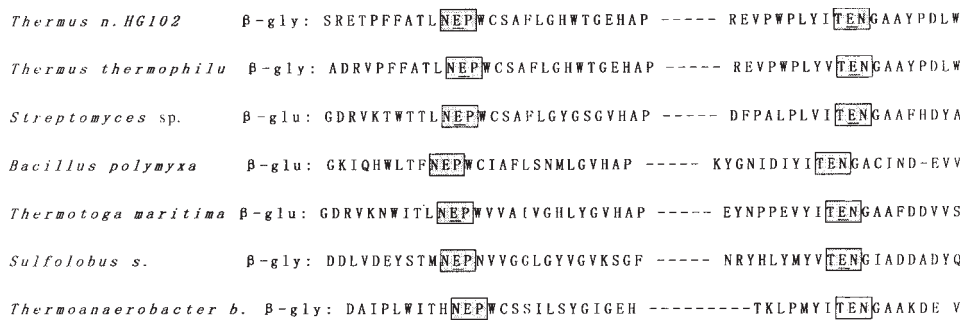

Fig. 5. Alignments of *T.n.-Gly* amino acid sequences with other bacterial origins. The two boxed sequences indicate the reserved motif in glycosyl hydrolase family I. The references were *T. thermophilus* β-glycosidase *(7)*, *Streptomyces* sp. β-glucosidase *(16)*, *B. polymyxa* β-glucosidase *(22)*, *T. maritima* β-glucosidase *(18)*, *S. solfataricus* β-glycosidase *(4)*, and *T. brockii* β-glycosidase *(17)*.

Table 1
Purification Procedure of *T.n.* β-Glycosidase

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Activity yield (%)
Crude extract	465.0	67,500	145	1.0	100.0
Heat precipitation	58.5	48,750	833	5.7	72.2
$(NH_4)_2SO_4$ (30–60%)	38.5	33,250	863	5.95	49.2
DEAE-cellulose DE52	7.9	16,000	2077	14.3	23.7
Prepared slab PAGE	1.2	6430	5358	36.9	9.5

step to remove most of the cellular proteins. Because some proteins cannot be removed using other methods, we used native prepared slab PAGE with activity staining (using pNPGalactopyranoside) with good results. Figure 6 shows the SDS-PAGE pattern of sample from each step of the purification. The purified recombinant β-glycosidase was electrophoretically homogeneous.

*Molecular Mass and p*I

The purified recombinant β-glycosidase was monomeric with a molecular mass of 48,900 Daltons as determined by SDS-PAGE and Superdex-75 gel filtration. These tests were in good agreement with that obtained from the amino acid sequence (48,997 Daltons). The p*I* of the purified enzyme was 5.2 as estimated from isoelectric focusing on PAG thin-layer gel (data not shown).

Enzymatic Reactions with Oligosaccharides

The enzymatic reactions with oligosaccharides were tested using cellobiose and lactose as substrates. *T.n.-Gly* exhibited only hydrolytic activity

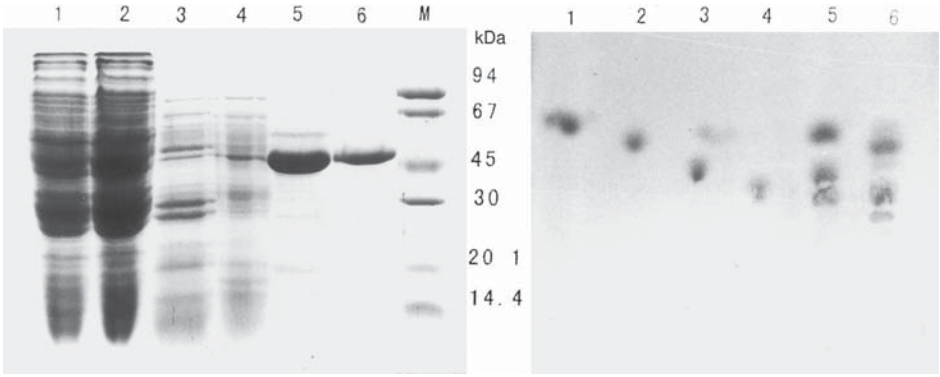

Fig. 6. *(left)* SDS-PAGE pattern of purification of *T.n.-Gly*. Lane 1, *E. coli* AS1.1739 (pUC18) cell-free extract; lanes 2–6, purification of *T.n.-Gly* from *E. coli* AS1.1739 (pHY); lane 2, cell-free extract; lane 3, supernatant of heat-treated extract; lane 4, supernatant after ammonium sulfate precipitation from 30 to 60% final saturation; lane 5, crude *T.n.-Gly* obtained from DEAE-cellulose chromatography; lane 6, *T.n.-Gly* obtained by prepared slab PAGE. M, standard marker proteins.

Fig. 7. *(right) T.n.-Gly* transglycosylation activity. Lanes 1–4, standard saccharides; lane 1, β-glucose; lane 2, β-galactose; lane 3, cellobiose; lane 4, lactose; lane 5, cellobiose reacted with *T.n.-Gly* at 65°C; lane 6, lactose reacted with *T.n.-Gly* at 65°C.

Table 2
Kinetic Parameters of *T.n.* β-Glycosidase

Substrate	K_m (m*M*)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
p-Nitrophenyl-β-D-glucoside	0.9	91.6	101.0
p-Nitrophenyl-β-D-fucoside	0.9	100.0	111.0
p-Nitrophenyl-β-D-galactoside	10.0	83.0	8.3
p-Nitrophenyl-β-D-mannoside	1.4	38.0	27.1

at a high temperature of 80°C. For a reaction temperature of 65°C with high substrate concentration, the transglycosylation activity was clearly detected (Fig. 7). We observed spots on the TLC plate that corresponded to oligosaccharides that were longer than reaction substrates.

Substrate Specificity and Kinetic Parameters

A variety of glycosides was tested for their ability to serve as substrates. *T.n-Gly* catalyzes the hydrolysis of β-D-galactosides (oNPGal, pNPGal, lactose), β-D-glucosides (oNPGlc, pNPGlc, cellobiose), β-D-fucosides (pNPFuc, oNPFuc), and β-D-mannose (pNPMan). Table 2 lists the kinetic parameters for pNPglycoside that were calculated. The k_{cat}/K_m ratio is a more significant parameter with respect to catalytic efficiency. The values for pNPFuc and pNPGlc were much higher than for pNPMan and pNPGal, and the value for pNPMan was higher than for pNPGal.

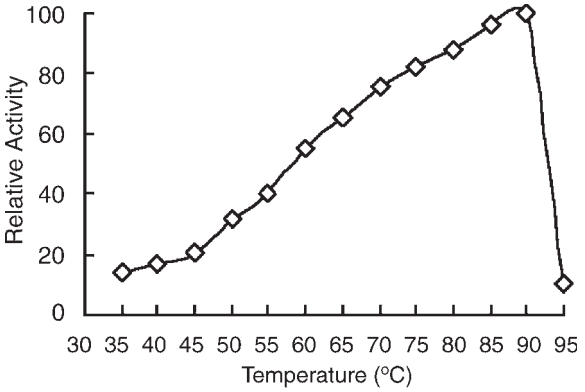

Fig. 8. Effect of temperature on the activity of *T.n.-Gly*. *T.n.-Gly* activity was determined as described in Materials and Methods at different temperatures from 35 to 95°C.

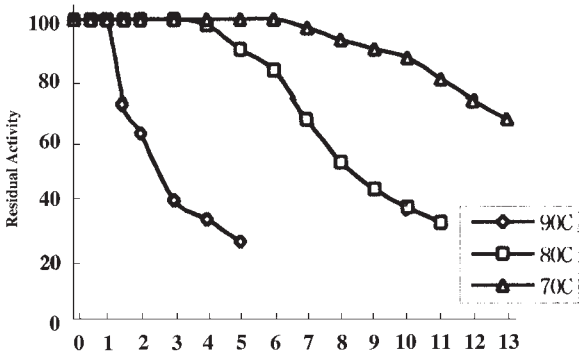

Fig. 9. Thermostability of *T.n.-Gly*. Purified enzyme was incubated at 70, 80, and 90°C for various times. The remaining activity was determined using standard method.

Effect of Temperature on Activity and Stability

The hydrolytic activities of the enzyme were determined at various temperatures from 35 to 95°C. The optimum temperature of the enzyme was 90°C (Fig. 8). The thermal stability of the recombinant enzyme was investigated by incubating the purified enzyme solution in a water bath at 70, 80, and 90°C for different times (Fig. 9). The residual activity was expressed as the percentage of the nonincubated enzyme activity. The half-life of the enzyme was 2.5 h at 90°C.

Effect of pH on Activity and Stability

The effect of pH on the activity was measured in different buffer systems with pH values from 3.0 to 10.0. The enzyme activity was measured by the standard method. The optimum pH for the enzyme was 5.6 (Fig. 10). To investigate the enzyme stability at varying pHs, the purified enzyme was exposed to different buffers of pH 3.0–10.0 for 30 min at 50°C. The

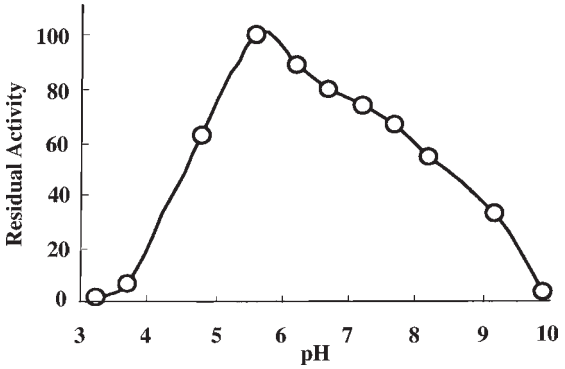

Fig. 10. Effect of pH on the activity of *T.n.-Gly*. *T.n.-Gly* activity was determined as described in Materials and Methods at different pHs from 4.0 to 10.0.

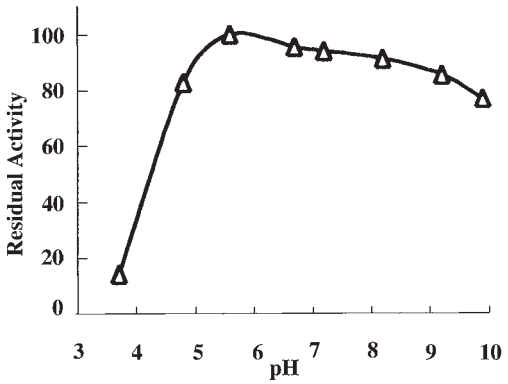

Fig. 11. pH stability of *T.n.-Gly*. Purified enzyme was incubated in different pH buffers at 50°C for 30 min. The remaining activity was determined using standard method.

remaining activity was expressed as the percentage of the untreated enzyme activity at pH 5.6. The enzyme was relatively stable within a pH range of 4.8–10.0, as indicated in Fig. 11.

Discussion

T. nonproteolyticus HG102 can produce several kinds of thermostable endoglycosidases. We have cloned and sequenced a β-glycosidase. The sequence revealed it was a member of the glycosyl hydrolase family I and shared a strong homology with other enzymes of the family that have been characterized. Enzymatic hydrolysis of the glycoside bond needs two critical residues: a proton donor and a nucleophile/base *(26)*. It has previously been shown that -N-E-P- and -T-E-N- are the critical sequences; the Glu of -N-E-P- is the proton donor (acid group), and the Glu of -T-E-N- is the nucleophile/base *(24,27,28)*. This suggests that the Glu164 and Glu338 may be the two important residues of *T. nonproteolyticus* HG102 β-glycosidase.

The deduced amino acid sequence and composition suggest reasons for the thermostability of *T. nonproteolyticus* HG102 β-glycosidase. First, the high Arg residue content (9.6% in comparison with an average occurrence of 5.1% in protein *[7]*) and the high Arg/Lys ratio might be important stabilizing factors in maintaining salt bridges at high temperatures. Folcarelli et al. *(29)* increased the protein thermostability by Lys → Arg mutations. Lys and Arg are always located on the surface of protein molecules and in contact with water. The hydrocarbon chain of Arg is one CH_2-group shorter than Lys and the bulky guanidine group of Arg may protect the hydrocarbon chains from unfavorable contact with water to enhance stability *(30)*. Second, the large proportion of Pro residues in the molecule (8.0% in comparison with an average occurrence of 5.2% in proteins *[7]*) may stabilize the protein molecule mainly by decreasing the entropy of unfolding. Watanabe et al. *(31)* reported that the high Pro content in amino acids is related to protein thermostability, especially when Pro is located at the second site of β-turns. Proline in the polypeptide chain has less conformation freedom than other amino acids, because the pyrrolidine ring of Pro imposes rigid constraints on the N–C_α rotation and restricts the available conformational space of the preceding residue of Pro *(32)*. Furthermore, Pro can bend the polypeptide onto itself to make the backbone much more amenable to hydrogen bonding with the polar side chains of other turn formers *(33)*, and the hydrophobic chain of Pro can interact with adjacent hydrophobic cavities, so that the turn would have a more fixed tertiary structure *(34)*. Third, a high content of α-helices (41.4%) in the predicted secondary structure agrees with the findings of Vieille and Zeikus *(35)*, which suggests that the α-helix had an important role in protein stability. Several single and multiple mutants that introduce residues with high helix propensity (such as Ala) can increase the enzyme's stability.

The β-glycosidase of *T. nonproteolyticus* HG102 can hydrolyze cellobiose and short oligosaccharides so that it can function as a cellobiase that can be involved in cellulose breakdown in synergy with endo- and exoglucanases. This research shows that the enzyme can synthesize oligosaccharides at high temperature, so it could be an attractive tool for the synthesis of oligosaccharides.

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