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Cloning and expression of a β-glycosidase gene from *Thermus thermophilus.* Sequence and biochemical characterization of the encoded enzyme

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A 3.2 kilobase pair DNA fragment from *Thermus thermophilus* HB27 coding for a β-galactosidase activity was cloned and sequenced. A gene and a truncated open reading frame orf1 encoding respectively a β-glycosidase (*ttβ-gly*) and probably a sugar permease were located directly adjacent to each other. The deduced aminoacid sequence of the enzyme Ttβ-gly showed strong identity with those of β-glycosidases belonging to the glycosyl hydrolase family 1. The enzyme was overexpressed in *Escherichia coli* and was purified by a two-step purification procedure. The recombinant enzyme is monomeric with a molecular mass of 49-kDa. It catalyzes the hydrolysis of β-D-galactoside, β-D-glucoside and β-D-fucoside derivatives. However, the kcat/Km ratio is much higher for p-nitrophenyl-β-D-glucoside and p-nitrophenyl-β-D-fucoside than for p-nitrophenyl-β-D-galactoside. The specificity towards linkage positions of the disaccharides tested decreased in the following order: β1-3 (100%) > β1-2 (71%) > β1-4 (40%) > β1-6 (10%). Ttβ-gly is a thermostable enzyme displaying an optimum temperature of 88°C and a half life of 10 min at 90°C. It performs transglycosylation reactions at high temperature with a yield exceeding 63% for transfucosylation reactions. On the basis of this work, the enzyme appears to be an attractive tool in the synthesis of fucosyl adducts and fucosyl sugars.

Keywords: **β-glycosidase gene, *Thermus thermophilus,* transglycosylation.**

Introduction

Structures and mechanisms of glycosyl hydrolases begin to be well-known [1]. These enzymes catalyze synthesis and transfer reactions as well as glycosidic linkage hydrolysis. They have been used for *in vitro* synthesis of oligosaccharides [2–4], various glycosidic derivatives [5], and mono- and disaccharide-amino acid conjugates [6–8]. Until now, the latter have always been obtained with low yields using the conventional sources of glycosidases (*Escherichia coli, Aspergillus oryzae,* almond, Jack bean). In this regard, glycosyl hydrolases from hyperthermophilic microorganisms are particularly attractive for their extreme stability and resistance to organic solvents [9] which can be useful properties for transglycosylation reactions. Several glucosidases and galactosidases have been purified or cloned from *Pyrococcus furiosus, Sulfolobus solfataricus* and *Thermotoga maritima* (for a review, see ref. [10]). The cloning of some genes has enabled high yields of pure protein to be obtained and structure/function relationships to be analyzed. For instance, the gene of β-glycosidase of *S. solfataricus* (Ssβ-gly) has been overexpressed [11] and the structure of the enzyme has been determined allowing a better explanation of its thermostability along with an accurate identification of the amino acids involved in the catalysis [12]. The transglycosylation properties of the recombinant enzyme have also been investigated at high temperature [13]. However the utilization of enzymes from hyperthermophiles is sometimes limited by difficulties in expressing the active form of the corresponding recombinant protein in the mesophilic *E. coli* host [9] or by their low activity at temperatures compatible with the stability of the substrates or products. Hence enzymes from thermophilic eubacteria, which are also highly resistant but display lower optimal temperatures, can be of great interest for many fields [14–16] and particularly for carbohydrate hydrolysis [17] and synthesis [18,19] since these molecules do not tolerate high temperatures.

Several glycosidase genes have already been cloned from thermophilic eubacteria and the encoded enzymes

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**The nucleotide sequence reported in this paper has been submitted to EMBL Nucleotide Sequence Library with accession number Y 16753

have been classified by Henrissat [20]. Although the *Thermus* genus is one of the most widely studied of the thermophilic ones, the β-glycosidases from this microorganism have not been extensively studied. An inducible β-galactosidase from *Thermus* 41-A strain has been isolated and only partially purified [21]. More recently, Berger *et al*. [18] demonstrated the presence of β-glycosidase activity in the crude extract of different strains of the *Thermus* genus including *T. thermophilus* HB8. β-Galactosidase activity in these strains was influenced in different ways by the variation of the aglycone moiety of the substrate suggesting the presence of several kinds of enzyme. Thus the β-galactosidase gene from *Thermus* T2 strain cloned by Koyama *et al*. [22] failed to hybridize with the *T. thermophilus* HB27 chromosome [23]. However, a weak and constitutive β-galactosidase activity detected in the HB27 strain [22] indicated that at least one β-galactosidase gene exists in this bacteria.

In the present study, we carried out the cloning and expression of a gene encoding a β-galactosidase from *T. thermophilus* HB27 in order to expand the basic knowledge about β-glycosidases from thermophilic bacteria and to use it for future structural studies and applications.

Materials and methods

Bacterial strains, plasmids and media

E. coli and *T. thermophilus* HB27 [24] strains were cultivated in LB medium at 37°C and 65°C respectively. Selection of ampicillin-resistant (100 μg/ml) *E. coli* was performed on LB agar plates. Expression of the *ttβ-gly* gene was carried out in *E. coli* TOP 10 (Invitrogen) and BL21(DE3) strain (Novagen) using plasmid vectors pUC18 and pET21a (Novagen) respectively.

Recombinant DNA techniques and cloning procedure

All the plasmid extractions were performed by alkaline lysis according to Ausubel *et al.* [25]. Restriction endonucleases, modifying enzymes, *Pfu* DNA polymerase and T4 DNA ligase were purchased from Boehringer Mannheim, Stratagene or Biolabs. For the genomic library, high molecular weight DNA from *T. thermophilus* was partially digested with *Sau3*A and size fractionated by agarose gel electrophoresis. The 3–20-kilobase pair fraction was extracted from the gel and ligated into *Bam*HI digested and dephosphorylated pUC18 vector [25]. The ligation mixture was used to transform *E. coli* TOP 10. Recombinant *E.coli* strains were cultivated and grown at 37°C to single colonies on the agar plates containing ampicillin (100 μg/ml). The plates were incubated at 60°C for 2h. Then 2 ml of Z buffer [26] containing 1.5 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was spread directly onto the colonies and the plates continued to be incubated at 60°C.

Overexpression and purification of the Ttβ-gly enzyme

The recombinant plasmid pLY12 was used for amplification of the *ttβ-gly* coding sequence with the *Pfu* DNA polymerase.

Two oligonucleotides bgl1: 5′-TA**CATATG**ACCGAGAACGCCGAAAA and bgl2: 5′-TT**GAATTC**TGGCGGGGCACTTAGG were used as primers and for the creation of *Nde* I and *Eco*RI restriction sites (in bold face) respectively at the extremities of the *ttβ-gly* gene by PCR. The amplified DNA was digested with *Nde*I and *Eco*RI and then inserted in the pET21a vector treated with the same enzymes. BL21(DE3) *E. coli* strain was transformed with the resulting pETBg8 plasmid and grown at 37°C, induced with 1mM isopropyl-β-D-thiogalactopyranoside (IPTG) at a cell density of 6×10^8 cells/ml and grown for another 4h. Cells were harvested by centrifugation, resuspended in 100 mM phosphate buffer (pH 7.0) and disrupted by sonication in a Raytheon sonicator (250W) for 2h at 25°C. The extract was centrifuged for 20 min at 6000 × g and the supernatant was incubated at 70°C for 1h. Labile proteins of *E. coli* were removed by centrifugation at 6000 × g for 20 min. The supernatant was concentrated by adding ammonium sulfate to 75% final saturation; then the precipitate was dissolved in 100 mM phosphate buffer (pH 7.0) and the resulting solution was passed through a Superdex-75 column (1.5 × 60 cm) that had been equilibrated with the same buffer, at a flow rate of 0.3 ml/min; fractions of 1ml were collected. The purity of the Ttβ-gly enzyme was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on continuous 10% polyacrylamide gels [27] and by capillary electrophoresis (P/ACE System 5000, Beckman).

DNA sequencing and analysis

The DNA sequence of the *ttβ-gly* gene was determined by Eurogentec on an AB1377-based fluorescent sequencing system using the pLY12 plasmid as template, synthetic primers and engineered thermophilic polymerase specifically dedicated for high accuracy DNA sequencing. Sequence comparisons with Genbank and Swissprot databanks were performed by means of the BLAST program. Precise alignments were performed by means of the ALIGN program located in « Pedro's Biomolecular Research Tools » on the World Wide Web.

Enzyme assay, kinetic parameters and protein determination

Under the standard test conditions, Ttβ-gly activity was measured at 65°C in 1 ml of 100 mM phosphate buffer (pH 7.0) containing 1 mM p-nitrophenyl-β-D-glucopyranoside (pNPGlc). A Kontron Uvikon 860 spectrophotometer, equipped with a cell holder system connected to a circulat-

ing water bath which maintained the cells at a constant temperature, was used. Cells of 1-cm path length were employed for all experiments. After prewarming the mixture for 5 min, the reaction was initiated by adding the enzymatic sample (5 µl) and the pNPGlc hydrolysis reaction was followed by measuring the increase in absorbance at 420 nm due to liberation of p-nitrophenoxide anion. The reference cell contained all reactants except the enzyme. Determination of other nitrophenyl-glycosidase activities was carried out in the same way using the corresponding p-nitrophenyl-glycopyranosides as substrates.

The kinetic parameters (Km and Vmax) were determined from Lineweaver-Burk and Eadie representations using different concentrations of p-nitrophenyl-β-D-glycopyranosides (0.05–15 mM) or cellobiose (5–100 mM). Each experimental point was determined at least in triplicate and in all cases the initial rate was used for plotting.

Enzyme activity on natural substrates was assayed in 1 ml of 100 mM phosphate buffer (pH 7.0) containing the tested substrate at the indicated concentration. After prewarming the mixture for 5 min at 70 °C, the reaction was initiated by 5 µl of the diluted enzyme solution. Aliquots (100 µl) were withdrawn at different times and the reaction was stopped by adding 4 µl of 10 M NaOH. The released glucose was measured by the glucose oxidase-peroxidase method [28]. Protein concentration was determined by the bicinchoninic acid method [29] using bovine serum albumin as the standard. The enzymatic unit was defined as the amount of enzyme that catalyzes the hydrolysis of 1 µmol of substrate per min at 65 °C under the conditions described above.

Temperature and pH optima, and thermal stability

The effect of pH on the enzyme activity was determined by measuring the hydrolysis of pNPGlc in a series of buffers at various pH values ranging from pH 4.1 to 9.1. The buffers used were: sodium acetate buffer (100 mM) from pH 4.1 to 5.1, sodium citrate/phosphate buffer (100 mM) from pH 5.0 to 7.2, sodium phosphate buffer (100 mM) from pH 6.0 to 8.0 and Tris-HCl buffer (100 mM) from pH 7.8 to 9.1. The pH values of each buffer were determined at 25 °C. The incubation mixture, prewarmed for 5 min at 70 °C, comprised 1 mM pNPGlc and 100 mM appropriate buffer in a total volume of 1 ml. The reaction was initiated by 5 µl of the diluted enzyme solution, carried out at 70 °C for 3 min and then stopped by adding 2 ml of 1 M Na_2CO_3. The absorbance at 420 nm was measured and the amount of liberated p-nitrophenoxide anion was determined from a calibration curve performed under the same experimental conditions.

The experimental protocol to study the dependence of the activity on temperature was the same as that for the pH-studies but the incubations were carried out at various temperatures ranging from 40°C to 95°C and only in 100 mM phosphate buffer at pH 7.0.

The thermal inactivation of the enzyme was studied at 70°C and 90°C by prewarming the enzyme solutions in 100 mM phosphate buffer (pH 7.0), removing aliquots at different times and measuring the residual activity under the standard conditions.

Transglycosylation reaction

The β-glycosyltransferase activity of Ttβ-gly was tested at 65°C in 3 ml of 100 mM phosphate buffer (pH 6.0) containing 0.1 unit of the enzyme, 10 mM p-nitrophenyl-β-D-glycopyranoside and 150 mM 2-phenylethanol used as the glycosyl acceptor. The progress of the reaction was monitored at different times (between 0.5 and 10 h) by withdrawing aliquots (200 µl) which were added to 400 µl of 1M sodium carbonate (pH 11.0). After filtration of the mixture through 0.45 µm hydrophilic Durapore membrane (Millipore), the reaction products (20 µl- portions) were analyzed by high-performance liquid chromotography (HPLC; Waters) on a LiChrospher 100RP-18 (5 µm) reverse-phase column (0.4 × 12.5 cm) from Merck. A methanol-water (35:65, v/v) mixture which was continuously purged with dry Helium was used as the eluent. Experiments were performed at room temperature and chromatographic separations were monitored by UV-absorption at 257 nm (2-phenylethyl- and p-nitrophenyl derivatives) and at 310 nm (p-nitrophenylderivatives), at a constant flow rate of 0.5 ml/min. Further characterization of the transglycosylation products was performed by NMR spectroscopy (Bruker WM250) [30].

Results

Cloning and nucleotide sequence of the ttβ-gly gene

As a β-galactosidase activity has already been observed in *T. thermophilus* HB27 [22], we tried to clone the corresponding gene(s). A genomic library of *T. thermophilus* HB27 was constructed in *E. coli* TOP10 by ligation of *Sau3*A incompletely digested DNA into *Bam*HI linearized pUC18 vector. Approximately 5000 recombinant clones containing inserts of 3- to 20-kilobase pairs were obtained and the plates were incubated at 60°C for 2h. At this high temperature *E. coli* cells die and *E. coli* β-galactosidase is inactivated, but if the thermostable *Thermus* enzyme is produced by the cells, β-galactosidase acivity can be detected. After addition of X-gal to the colonies and further incubation at 60°C, one clone turned blue. The recombinant plasmid, designated pLY12, was extracted from this dead colony and used to retransform *E. coli* TOP 10. pLY12 contained a 3.2-kilobase pair insert as shown by a restriction map analysis.

The entire sequence of the DNA insert was determined and the 1949-base pair sequence located downstream of the

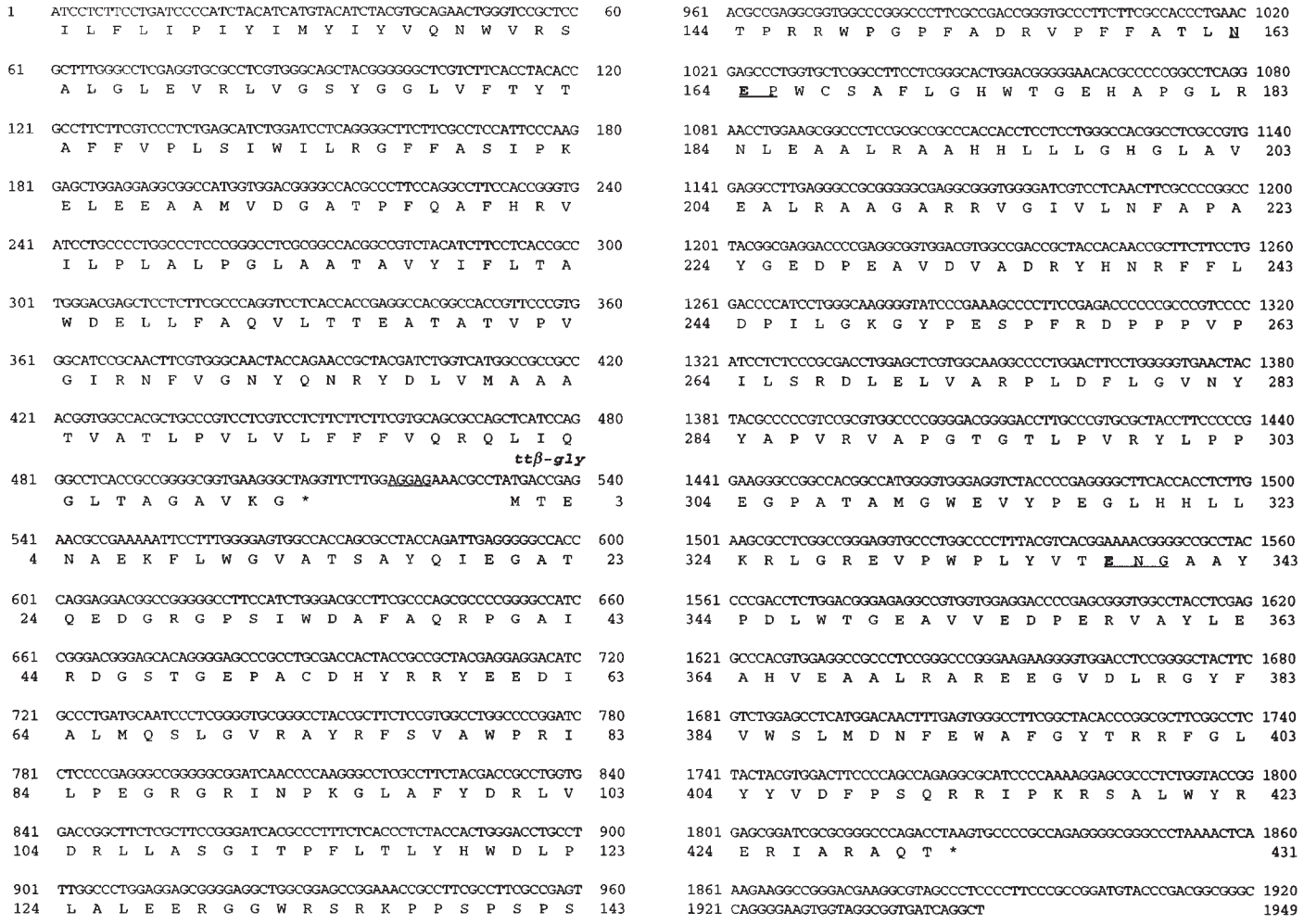

Figure 1. Nucleotide and deduced amino acid sequences of the *ttβ-gly* gene and of the truncated orf1. (EMBL accession number Y16753). Numbering of the nucleotide sequence begins at the *Bam*HI/*Sau*3A junction site beside the lactose promoter. Numbering of the amino acid sequence begins at the Ttβ-gly amino-terminus. The putative ribosome binding site of the *ttβ-gly* gene is underlined. The two amino acid motifs fully conserved among glycosyl hydrolases from thermophilic and mesophilic bacteria are also underlined. The putative nucleophile (Glu338) and general acid base catalyst (Glu164) are represented in bold character.

lactose promoter is presented in Figure 1. Its overall G+C content was 69.3%, which is very close to that of the DNA of the whole *T. thermophilus* HB8 genome (69%) [31]. Computer analysis revealed the presence of a 1296-base pair orf displaying strong homologies with several β-glucosidase genes. The other part of this insert (data not shown) did not display significant homology with already known glycosidase genes. It appeared likely therefore that the 1296-base pair orf encoded for a β-glucosidase which also displayed a β-galactosidase activity that we could detect on the colony. This point has been biochemically confirmed (see below) and this orf was therefore designated as being a glycosidase gene of *T. thermophilus*, termed *ttβ-gly*. This enzyme should be 431 amino acids long with a deduced molecular mass of 48642 Da. The ATG initiation codon is preceded by a typical ribosome binding site of *E. coli* but no typical promoter sequence of *T. thermophilus* [32] was detected upstream of the *ttβ-gly* orf. Thus the thermostable glycosidase expression was probably due to the lactose promoter correctly oriented and located at 0.6-kilobase pair upstream of the *ttβ-gly orf.* Between these two sequences, a 511-base pair truncated orf (designated orf1) was found presenting significant similarities (41 to 54%) with several sugar permease genes. No palindromic sequence was detected between the orf1 and ttβ-gly sequences.

Sequence comparison between the Ttβ-gly protein and other glycosidases

The amino acid sequence deduced from the encoded Ttβ-gly from *T. thermophilus* HB27 was compared with other glycosyl hydrolases whose sequences are available in data-

bases. This analysis revealed that the enzyme is a member of the glycosyl hydrolase family 1 [20] which is composed in particular of β-glucosidases, phospho-β-glucosidases, and phospho-β-galactosidases of Bacteria, Archea, and Eucarya. Scores higher than 40% of identity over the entire length of sequences were found between Ttβ-gly and the β-glycosidases of i) thermophilic bacteria: 45.5% with BglA of *T. maritima* [33], 43.8% with BglA of *Caldocellum saccharolyticum* [34], 45.6% with BglB of *Microbispora* [35] and of ii) mesophilic bacteria: 48.2% with Bgl3 of *Streptomyces* sp. [36], 44.3% with Abg of *Agrobacterium* sp. [37]. Scores were much lower with β-glycosidases of Archea and Eucarya: 27.1% and 28.8% with the β-glycosidases Ssβ-gly of *S. solfataricus* [38] and CelB of *P. furiosus* [39] respectively, 28.8% with the cyanogenic β-glucosidase Cbg of *Trifolium repens* [40]. The two motifs Asn-Glu-Pro and Glu-Asn-Gly, which are fully conserved among glycosyl hydrolases from mesophilic and thermophilic bacteria as described by Morracci *et al*. [41], were also conserved in the Ttβ-gly sequence (Figure 1). The Glu338 located in the second motif of the *T. thermophilus* sequence would correspond to the nucleophile identified previously in the active sites of Abg [42,43], Ssβ-gly [41], Cbg [44], and the phospho-β-galactosidase of *Lactococcus lactis* (LacG) [45]. The Glu164 located in the first motif would correspond to the general acid/base catalyst identified previously in the active sites of Abg, Ssβ-gly, Cbg, and LacG.

Overexpression and purification of the Ttβ-gly enzyme

For the expression of the recombinant *T. thermophilus* enzyme, we used the T7 RNA polymerase gene expression system [46]. The *ttβ-gly* coding sequence was amplified by PCR and cloned in the pET21a expression vector to obtain the pETBg8 plasmid. A cell sample of IPTG-induced *E. coli* BL21 (DE3)/pETBg8 was analyzed by SDS-PAGE (Figure 2). A major band corresponding to a 50-kDa protein and representing about 50% of total proteins was detected in the crude extract. Ttβ-gly enzyme was purified by heat treatment of the cell-free extract allowing the precipitation of the majority of *E. coli* proteins (Figure 2). A last step on Superdex-75 column achieved the purification of the enzyme. A sample of the purified enzyme was analyzed by capillarity electrophoresis and displayed a single peak indicating the homogeneity of the preparation.

Molecular properties

The molecular weight of 48642 deduced from the amino acid sequence is in good agreement with that obtained by electrophoresis on polyacrylamide gels in the presence of SDS (Figure 2) and also with that determined by gel-filtration through a calibrated Superdex-75 column in the absence of any denaturing agent. The fact that denatured and non-denatured Ttβ-gly exhibit the same molecular weight suggests a monomeric structure for the enzyme. Its theoretical isoelectric point was found to be 6.47 as estimated using the DNASIS program.

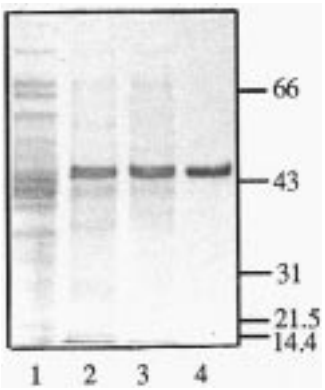

Figure 2. SDS-PAGE of Ttβ-gly expressed in *E. coli.* The gel was stained with Coomassie Brilliant Blue; lane 1, crude cell extract of BL21/pET21a; lanes 2–4, purification of Ttβ-gly from IPTG-induced *E.coli* BL21(DE3)/pETBg8 : lane 2, crude cell extract; lane 3, supernatant of heat-treated extract; lane 4, Ttβ-gly obtained after filtration on Superdex-75 column. Molecular masses of standard proteins (values in kDa) are indicated at the right.

The relative degree of the polar or non-polar character of a protein depends on the proportion of both hydrophilic (H) and apolar (A) sets of amino acids. We have classified Asx,Glx, Lys, Arg, Ser, Thr and His as hydrophilic residues (H) and Val, Ile, Leu, Phe and Met as apolar residues (A). This classification follows that proposed by Hatch and Bruce [47]. The H/A ratio, calculated from the amino acid composition, is 1.5. This value is lower than that of most soluble proteins (mean value, H/A = 2.1) and indicates a relatively large proportion of apolar residues. It is also interesting to note the large content of Pro (9.5%) and Arg (10.2%), and the high Arg/Lys ratio (7.3).

Physico-chemical properties

The effect of pH on the catalytic activity of the enzyme was studied at 70 °C between pH 4.1 and 9.1. The pH/activity curve displayed a maximum at pH 6.5–7.0 (Figure 3). The enzyme retained more than 80% of its activity in the range pH 6.0–7.5 and at least 50% at pH 4.1 and 8.4. An inhibitory effect of Tris was observed on the enzyme activity. This amine buffer is a well-known inhibitor of many glycosidases [48] and cannot be considered as either inert or unreactive in many enzyme reactions [49].

The optimum temperature for the hydrolysis of pNPGlc was 88°C (Figure 4) and the value of the temperature coefficient (Q_{10}) calculated between 70°C and 80°C was 1.5. The

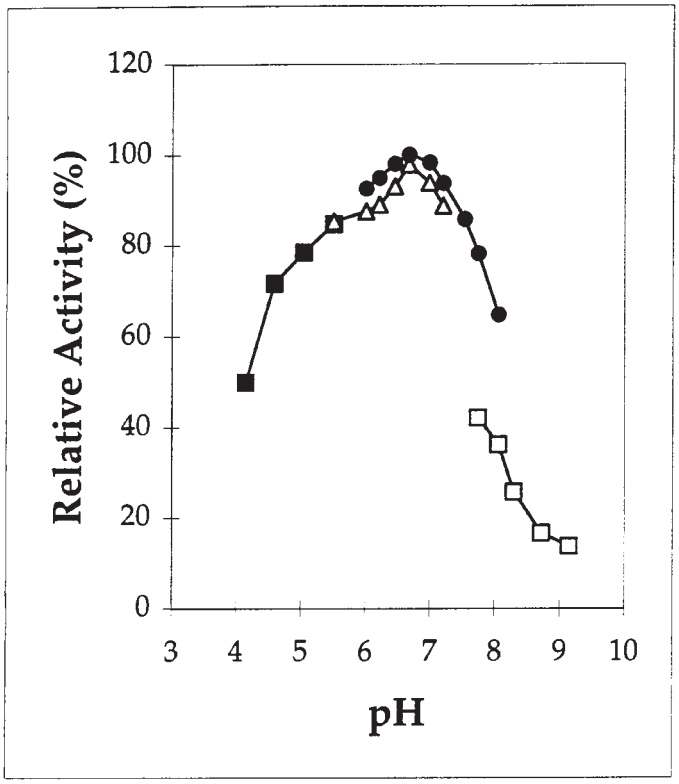

Figure 3. Effect of pH and buffer on Ttβ-gly activity. The experiments were carried out at 70°C with pNPGlc as substrate and using sodium acetate (■), sodium citrate/phosphate (△), sodium phosphate (●), and Tris-HCl(□) buffers; for further details, see « Materials and Methods ».

latter is much lower than that observed for most enzymes (Q_{10} around 2.0). From the Arrhenius plot (inset Figure 4) the activation energy was found to be 38.2 kJ/mol. The effect of temperature on the enzyme stability was also investigated by incubating enzyme solutions for 10 min at different temperatures (Figure 5). The enzyme retained 100% of its activity up to 80°C. At higher temperatures, thermostability decreased rapidly : the half-life of the enzyme was 10 min at 90°C and it completely lost its activity after treatment at 98°C for 10 min.

Substrate specificity and kinetic parameters

A variety of glycosides were tested for their ability to serve as substrates. Ttβ-Gly is an exoglycosidase with a high specificity for the β-anomeric configuration of the glycosidic linkage. It catalyzes the hydrolysis of β-D-galactosides (oNPGal, pNPGal, lactose), β-D-glucosides (oNPGlc, pNPGlc, cellobiose) and β-D-fucosides (oNPFuc, pNPFuc). The enzyme can also hydrolyze β-D-xylosides but at a much lower rate. No N-acetyl-β-D-galactosaminidase or N-acetyl-β-D-glucosaminidase activity was detected using the corresponding p-nitrophenyl derivatives as substrates. For purposes of comparison, the kinetic parameters were determined, in particular, for three substrates (derivatives of β-D-galactoside, β-D-glucoside, and β-D-fucoside) carrying the same aglycone moiety (p-nitrophenyl) in order to eliminate the potential effect of the latter on their kinetic behavior. Examination of Table 1 shows that the enzyme specificity is largely dependent on the nature of the substrate tested. The Km values indicate a high binding specificity for pNPGlc, the relative affinity for glycosyl residues decreases in the following order: β-D-glucosyl > β-D-fucosyl > β-D-galactosyl. Although the value of kcat is in favor of pNPGal, the kcat/Km ratio, known to be the more significant parameter with respect to catalytic efficiency, is much higher for pNPlc and pNPFuc than for pNPGal.

Concerning the specificity towards linkage positions, the enzyme preferentially cleaves the β1-3 bond. The rate of hydrolysis of the disaccharides tested at a concentration of 5 mM decreased in the following order : Glc β1-3 Glc (laminaribiose) (100% reaction rate) > Glc β1-2 Glc (sophorose) (71%)> Glc β1-4 Glc (cellobiose) (40%)> Glc β1-6 Glc (gentiobiose) (10%). In addition to disaccharides, the enzyme was able to hydrolyze oligosaccharides. The hydrolysis rate, measured by the amount of glucose released in the reaction medium, increased with the chain length : cellopentaose (100%)> cellotetraose (87.5%) >cellotriose (60%).

Evidence for the hydrolysis of β-D-galactosides, β-D-glucosides and β-D-fucosides at a common catalytic site

The presence on the enzyme of a common site responsible for the hydrolysis of β-D-galactosides, β-D-glucosides and β-D-fucosides was tested by experiments of mutual competition between substrates and studies of thermal inactivation. First, mixed-substrate incubations involving two p-nitrophenyl-β-D-glycosides were performed and the results were treated by the Dixon method [50]. As shown in Table 2, the values observed for the total velocity of hydrolysis agreed quite well with the theoretical ones calculated from the common site reaction and disagreed with those calculated from a reaction scheme involving distinct sites (one per substrate). Secondly, thermal inactivation studies, performed at 70°C and 90°C, displayed that the β-D-galactosidase, β-D-glucosidase and β-D-fucosidase activities were lost in the same way in the course of time (Figure 6). The enzyme inactivation kinetics showed first-order reactions with values of the rate constant estimated to be 0.08 h^{-1} and 0.09 min^{-1} at 70°C and 90°C respectively. Finally, both experiments indicated a common catalytic site at which all tested substrates were hydrolyzed.

Transglycosylation reactions

The ability of Ttβ-gly to catalyze transglycosylation reactions was tested with p-nitrophenyl-β-D-glycopyranosides as glycosyl donors and 2-phenylethanol as a glycosyl ac-

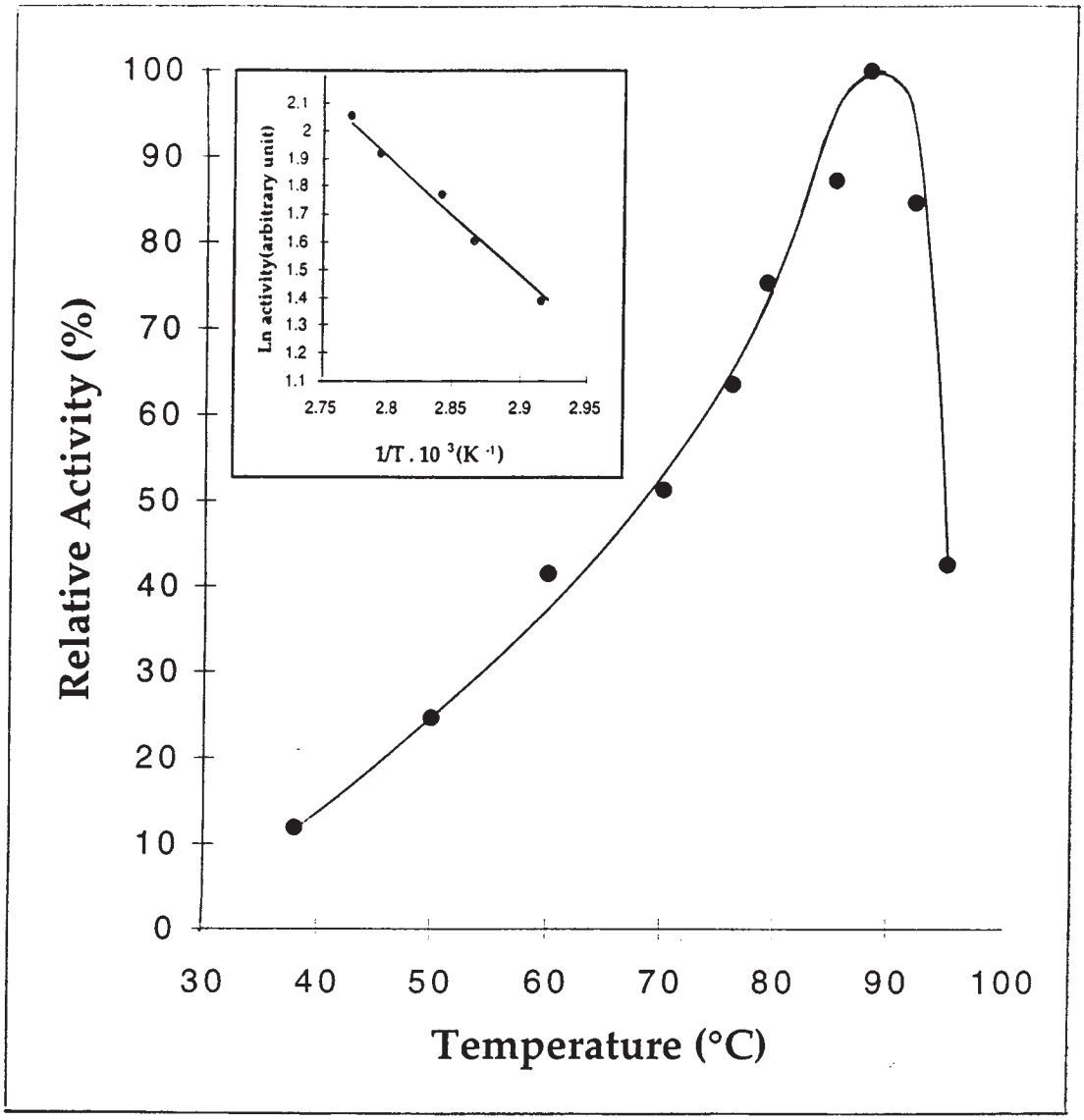

Figure 4. Effect of temperature on Tt β-gly activity. The experiments were carried out at the indicated temperatures for 3 min in 100 mM sodium phosphate buffer (pH 7.0) containing 1mM pNPGlc; for further details, see « Materials and Methods ». Inset: Arrhenius plot of the data in the range 70–85 °C.

ceptor. The latter has already been used to test the transglycosylase activities of β-galactosidase from *Aspergillus oryzae* [51] and *Achatina achatina* [8,52]. Glycosylation kinetics were studied as a function of incubation time and are reported in Figure 7 for the 2-phenylethyl-β-D-fucoside synthesis. The yields of the transglycosylation products depended on the glycosyl donor; pNPFuc gave a two-fold higher yield than pNPGal and pNPGlc (Table 3).

Discussion

Screening of an expression library of *T. thermophilus* HB27 with the X-gal chromogenic substrate gave rise to a clone displaying a thermostable β-galactosidase activity. The sequence of the cloned DNA fragment revealed the presence of a gene sharing a strong homology with already characterized β-glucosidases of the glycosyl hydrolase family 1, according to the classification based upon amino acid sequence similarities [20]. The sequence also showed that the orf 1 located upstream of the *ttβ-gly* orf very probably encodes a sugar permease and seems to belong to the same transcription unit as that of *ttβ-gly* since neither a typical promoter of *T. thermophilus* nor a putative transcriptional terminator was found between these two close orfs. Thus these genes display the classical structure of operons for β-glycoside utilization as it has been observed in *E. coli, B. subtilis* and other eubacteria. However, this point should be confirmed since in the hyperthermophilic microorganism *S.*

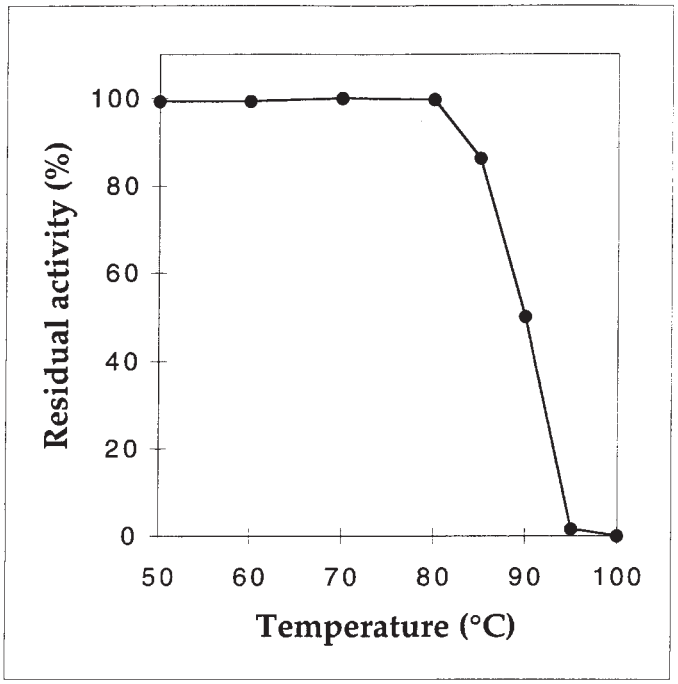

Figure 5. Thermal denaturation of Ttβ-gly. The enzyme was maintained for 10 min at the indicated temperatures in 100 mM sodium phosphate buffer (pH 7.0). The residual activity was then measured under the standard assay conditions.

solfataricus, the same genetic organization led to the transcription of the permease and glycosidase genes from independent promoters [53].

The high thermostability of the recombinant β-glycosidase allowed its complete purification from *E. coli* /pETBg8 and its biochemical and molecular characterization. Ttβ-gly is a monomeric enzyme with a molecular mass of 48642Da. Although most subunits of family 1 glycosyl hydrolases have molecular masses in the range of 45 to 60 kDa, the thermophilic β-glucosidases belonging to this family differ in their subunit structure which can be monomeric like BglA from *C. saccharolyticum* [38] and *Clostridium thermocellum* [54], and CglT from *Thermoanaerobacter brockii* [17], dimeric like BglA from *T. maritima* [55] or tetrameric like Ssβ-gly from *S. solfataricus* [56] and CelB from *P. furiosus* [57].

Table 1. Kinetic parameters of the *Thermus thermophilus* β-glycosidase

Substrate	*Km (mM)*	k_{cat} (s^{-1})	k_{cat}/Km ($mM^{-1}s^{-1}$)
p-nitrophenyl-β-D-galactoside[a]	6.6	118	18
p-nitrophenyl-β-D-glucoside[a]	0.09	23	256
p-nitrophenyl-β-D-fucoside[a]	0.16	56	347
Cellobiose[a,b]	4.0	41	10

a. The experiments were carried out at 65°C for the p-nitrophenyl-β-D-glycosides and at 70 °C for cellobiose
b. For cellobiose, the value of kcat was calculated taking into account that one mol of cellobiose liberated two mol of glucose.

Ttβ-gly displayed a broad substrate specificity as is the case with most glycosyl hydrolases belonging to family 1. The enzyme was able to catalyze the hydrolysis of various β-galactoside, β-glucoside and β-fucoside derivatives. As demonstrated by mutual competition experiments between substrates and studies of thermal inactivation, all the hydrolyzed substrates were at least recognized by a common catalytic site. The Ttβ-gly hydrolytic activity was not limited to a single linkage type. Indeed, although the enzyme cleaved preferentially the β1-3 bond, it hydrolyzed, at an appreciable rate, disaccharides with β1-2 and β1-4 linkages but very weakly the β1-6 linkage. Such a linkage specificity was also found for recombinant *T. brockii* β-glucosidase CglT purified from *E. coli* [17] and for Ssβ-gly [58] although for the latter the β1-2 bond was not tested. Concerning the enzyme activity in relation to oligosaccharide chain length, Ttβ-gly hydrolyzed short-chain cello-oligosaccharides up to at least pentamers, but the enzyme was practically inactive on high molecular weight polymers such as laminarin. In this case, Ttβ-gly showed the same behavior as β-glucosidase purified from barley seeds [59], while for Ssβ-gly the maximum catalytic efficiency was observed with cellotetraose and hydrolysis was lower with cellopentaose.

Enzymatic hydrolysis of the β-glycosidic bond requires the presence of two critical residues: a proton donor and a nucleophile/base [60]. Members of family 1 catalyze the transfer of glucosyl groups between oxygen nucleophiles with retention of the anomeric configuration at the C1 position, the nucleophile catalytic base being close to the sugar anomeric carbon (1). The two glutamic residues, essential for catalysis in Ttβ-gly, were presumed to be Glu164 and Glu338 as determined from sequence alignments with other glycosidases of the glycosyl hydrolase family 1 [41–45]. Mutagenesis experiments are in progress in order to confirm the catalytic role of these amino acids.

There is little information available on the enzymatic synthesis of oligosaccharides and glycoconjugates by enzymes from thermophilic bacteria. Only a few studies have been performed on glycoside derivative production at high temperatures [13,18,61–63] and no study has been reported on the transfucosylase activity of thermozymes. However, work has been carried out by Nunoura *et al.* [64] with a β-D-glucosidase (β-D-fucosidase), stable up to 40°C, from *Bifidobacterium breve* clb acclimated to cellobiose. The enzyme displayed transferase activity for the β-D-fucosyl group but not for the β-D-glucosyl group. An interesting property of Ttβ-gly is also its glycosyltransferase activity which was quantitatively measured in the present study. The enzyme was capable of transferring β-D-galactosyl, β-D-glucosyl and β-D-fucosyl groups from corresponding

Table 2. Mixed-substrate analysis on p-nitrophenyl-β-D-glycopyranosides. Studies were made using three different combinations of substrates as follows: pNPGal + pNPGlc, pNPGlc + pNPFuc and pNPGal + pNPFuc. The values calculated by a simple summation of the hydrolytic rate of each substrate and the values calculated assuming a common site were determined by the Dixon equation [50].

			Rate of p-nitrophenoxide ions formed (arbitrary units)		
			Calculated		
pNPGal (7 mM)	*pNPGlc (0.1 mM)*	*pNPFuc (0.2 mM)*	*Distinct sites*	*Common site*	*Observed*
0.718	0.149		0.867	0.565	0.598
	0.149	0.284	0.433	0.284	0.282
0.718		0.284	1.002	0.640	0.667

p-nitrophenyl-β-D-glycosides but the most surprising feature of Ttβ-gly was its very high level of transfucosylase activity (63 % after 4 h-reaction). On the basis of these results, Ttβ-gly could be used as an attractive tool for the synthesis of fucosyl adducts or dissaccharides such as fucosyl sugars [64].

The structural basis of the thermostability of enzymes from thermophilic organisms is still poorly understood although most protein-stabilization mechanisms implicated in mesozymes are also found in thermozymes (for a review, see ref. [65]). The enhanced stability of thermozymes must be a result of differences in specific amino acid compositions and sequences [65]. First, the large contribution of apolar residues to the total amino acid composition of Ttβ-gly suggests that this enzyme is probably stabilized by intramolecular hydrophobic interactions in the protein core. Secondly, the high content of Arg residues (10.2 % in comparison with 5.1 %, the average occurence in proteins) and

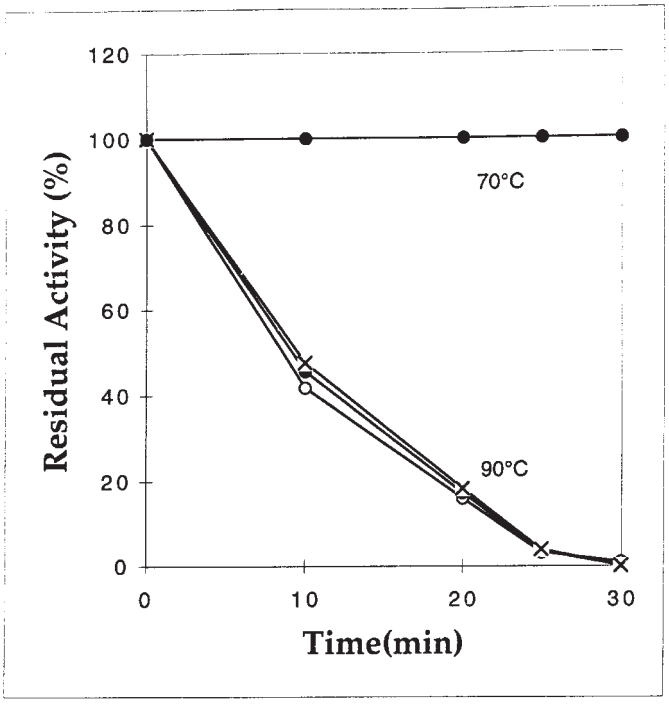

Figure 6. Thermal inactivation of Ttβ-gly. The enzyme was preincubated at 70°C and 90°C in 100mM sodium phosphate buffer (pH 7.0). At the indicated times, aliquots were withdrawn and the residual activities were measured at 65°C in the same buffer using pNPGal (○), pNPGlc (●), and pNPFuc (x) as substrates.

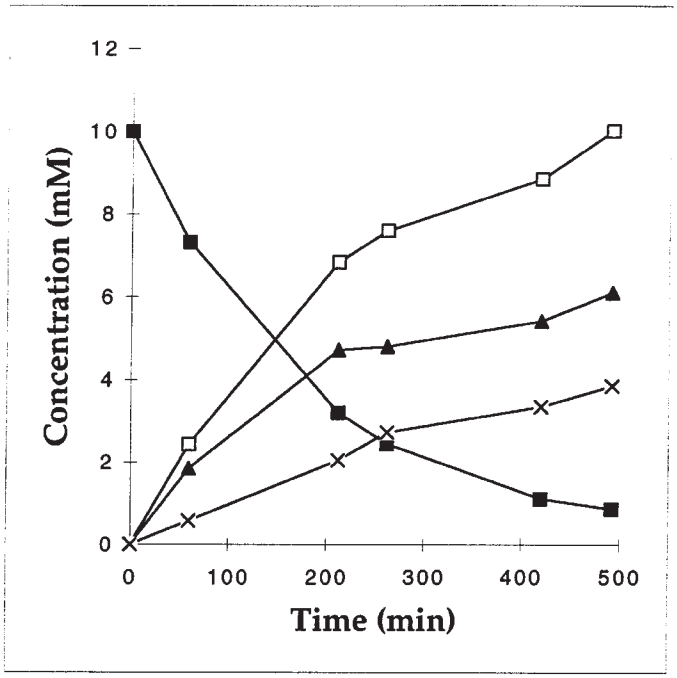

Figure 7. Time course of 2-phenylethyl-β-D-fucoside synthesis by Ttβ-gly. The transfucosylation activity was tested at 65°C in 3 ml of 100 mM sodium phosphate buffer at pH 6.0 containing 10 mM p-nitrophenyl-β-D-fucoside, 150 mM 2-phenylethanol and 0.1 unit of Ttβ-gly. The different products were analyzed and quantified as described under « Materials and Methods ». Symbols pNPFuc (■), pNP (□), PEFuc (▲), fucose (X).

Table 3. Transglycosylation reactions with Ttβly. The enzymatic reaction was carried out with 10 mM of pNPGal, pNPGlc or pNPFuc, 150 mM of PE and 0.1 unit of Ttβly in 3 ml of 100 mM phosphate buffer (pH 6). After incubation at 65 °C for 4 h, the hydrolysis and transglycosylation products were quantified by HPLC ; for further details, see "Materials and Methods".

Substrate	*Initial concentration mM*	*pNP formed mM*	*Hydrolysis (%)*	*Transglycosylation (%)*
pNPGal	10	4.1	69	31
pNPGlc	10	4.6	65	35
pNPFuc	10	7.6	37	63

the high Arg/Lys ratio in Ttβ-gly might be an important stabilizing factor in maintaining salt-bridges at high temperatures [66]. Thirdly, the large proportion of Pro residues in the Ttβ-gly molecule (9.5 % in comparison with 5.2 %, the average occurence in proteins) may stabilize the protein molecule mainly by decreasing the entropy of unfolding [67].

Concerning the possible *in vivo* role of Ttβ-gly, its hydrolytic activity towards cellobiose and short cellooligosaccharides suggests that it functions as a cellobiase which could be involved in cellulose breakdown in synergy with endo- and exo-glucanases. The cellodextrins could be imported by the orf1-encoded-sugar permease since orf1 and *ttβ-gly* probably belong to the same operon. However, it cannot be excluded that the strong β-1,3-hydrolytic activity of Ttβ-gly is involved in the terminal laminarin degradation, as seems to be the case for the CelB glucosidase of *P. furiosus* (39), or in other β-1,3-glycoside hydrolyses.

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References

1 Davies G, Henrissat B (1995) *Structure* **3**: 853–59.
2 Cote G L, Tao BY (1990) *Glycoconjugate J* **7**: 145–62.
3 Ichikawa Y, Look GC, Wong C-H (1992) *Anal Biochem* **202**: 215–38.
4 Yoon J-H, Ajisaka K (1996) *Carbohydr Res* **292**: 153–63.
5 Nilsson, KGI (1991) In *Enzymes in Carbohydrate Synthesis* (Bednarski MD, Simon E S, eds) pp 51–62, ACS Symposium Series 466, Washington, D.C.
6 Attal S, Bay S, Cantacuzene D (1992) *Tetrahedron* **48**: 9251–60.
7 Leparoux S, Padrines M, Fortun Y, Colas B (1996) *Biotechnol Lett* **18**: 135–38.
8 Leparoux S, Padrines M, Placier G, Colas B (1997) *Biochim Biophys Acta* **1336**: 522–32.
9 Adams M W W, Perler F B, Kelly RM (1995) *Bio/technology* **13**: 662–68.
10 Bauer MW, Halio, SB, Kelly RM (1996) *Adv Protein Chem* **48**: 271–310.
11 Moracci M, Nucci R, Febbraio F, Vaccaro C, Vespa N, La Cara F, Rossi M (1995) *Enzyme Microb Technol* **17**: 992–97.
12 Aguilar CF, Sanderson I, Moracci M, Ciaramella M, Nucci R, Rossi M, Pearl LH (1997) *J Mol Biol* **271**: 789–802.
13 Trincone A, Improta R, Nucci R, Rossi M, Gambacorta A (1994) *Biocatalysis* **10**: 195–210.
14 Dion M, Loussouarn F, Batisse N, Rabiller C, Sakanyan V (1995) *Biotechnol Lett* **17**: 905–10.
15 Lee SG, Lee DC, Kim HS (1997) *Appl Biochem Biotechnol* **62**: 251–66.
16 Saiki RK, Gelfand DH, Stoffel S, Scharf SJ, Higuchi R, Horn GT, Mullis KB, Erlich HA (1988) *Science* **239**: 487–91.
17 Breves R, Bronnenmeier K, Wild N, Lottspeich F, Staudenbauer WL, Hofemeister J (1997) *Appl Environ Microbiol* **63**: 3902–10.
18 Berger J-L, Lee BH, Lacroix C (1995) *Appl Microbiol Biotechnol* **44**: 81–87.
19 Nakao M, Nakayama T, Harada M, Kakudo A, Ikemoto H, Kobayashi S, Shibano Y (1994) *Appl Microbiol Biotechnol* **41**: 337–43.
20 Henrissat B, (1991) *Biochem J* **280**: 309–16.
21 Cowan DA, Daniel RM, Martin AM, Morgan HW (1984) *Biotechnol Bioeng* **26**: 1141–45.
22 Koyama Y, Okamoto S, Furukawa K (1990) *Appl Environ Microbiol* **56**: 2251–54.
23 Tabata K, Hoshino T (1996) *Microbiology* **142**: 401–10.
24 Sakaki Y, Oshima T (1975) *J Virol* **15**: 1449–53.
25 Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1993) *Current Protocols in Molecular Biology.* New York: John Wiley.
26 Miller JH (1992) *A Short Course in Bacterial Genetics.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
27 Laemmli UK (1970) *Nature* **227**: 680–85.
28 Kunst A, Draeger B, Ziegenhorn J (1984) In *Methods of Enzymatic Analysis* (Bergmeyer H U, ed) vol 6, pp 178–85. New York and London: Verlag Chemie, Weinheim and Academic Press.
29 Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) *Anal Biochem* **150**: 76–85.
30 Leparoux S, Fortun Y, Colas B (1994) *Biotechnol Lett* **16**: 677–82.
31 Oshima T, Imahori K (1974) *Int J Syst Bacteriol* **24**: 102–12.
32 Maseda H, Hoshino T (1995) *FEMS Microbiol Lett* **128**: 127–34.
33 Lieb W, Gabelsberger J, Schleifer K-H (1994) *Mol Gen Genet* **242**: 111–15.
34 Love DR, Fisher R, Bergquist PL (1988) *Mol Gen Genet* **213**: 84–92.

35 Wright RM, Yablonsky MD, Shalita ZP, Goyal AK, Eveleigh DE (1992) *Appl Environ Microbiol* **58**: 3455–65.
36 Perez-Pons JA, Cayetano A, Rebordosa X, Lloberas J, Guasch A, Querol E (1994) *Eur J Biochem* **223**: 557–65.
37 Wakarchuk WW, Greenberg NM, Kilburn DG, Miller RC Jr, Warren RAJ (1988) *J Bacteriol* **170**: 301–07.
38 Cubellis MV, Rozzo C, Montecucchi P, Rossi M (1990) *Gene* **94**: 89–94.
39 Voorhorst WG, Eggen RI, Luesink EJ, de Vos WM (1995) *J Bacteriol* **177**: 7105–11.
40 Oxtoby E, Dunn MA, Pancoro A, Hughes MA (1991) *Plant Mol Biol* **17**: 209–19.
41 Moracci M, Cabaldo L, Ciaramella M, Rossi M (1996) *Protein Eng* **9**: 1191–95.
42 Withers SG, Warren RAJ, Street IP, Rupitz K, Kempton JB, Aebersold R (1990) *J Am Chem Soc* **112**: 5887–89.
43 Wang Q, Trimbur D, Graham R, Warren RAJ, Withers SG (1995) *Biochemistry* **34**: 14554–62.
44 Barret T, Suresh CG, Tolley SP, Dodson EJ, Hughes MA (1995) *Structure* **3**: 853–59.
45 Wiesmann C, Beste G, Hengstenberg W, Schulz GE (1995) *Structure* **3**: 961–68.
46 Studier FW, Moffat BA (1986) *J Mol Biol* **189**: 113–30.
47 Hatch FT, Bruce AL (1968) *Nature* **218**: 1166–68.
48 Colas B (1980) *Biochim Biophys Acta* **613**: 448–58.
49 Mahler HR (1961) *Ann NY Acad Sci* **92**: 426–39.
50 Dixon M, Webb EC (1979) *Enzymes,* 3rd ed., pp 72–75. London: Longman Group Limited.
51 Fortun Y, Colas B (1991) *Biotechnol Lett* **13**: 863–66.
52 Leparoux S, Colas B (1994) *Int J Biochem* **26**: 247–54.
53 Prisco A, Moracci M, Rossi M, Ciaramella M (1995) *J Bacteriol* **177**: 1614–19.
54 Gräbnitz F, Seiss M, Rücknagel KP, Standenbauer WL (1991) *Eur J Biochem* **200**: 301–09.
55 Gabelsberger J, Liebl W, Schleifer K-H (1993) *FEMS Microbiol Lett* **109**: 131–38.
56 Pisani FM, Rella R, Raia C, Rozzo C, Nucci R, Gambacorta A, De Rosa M, Rossi M (1990) *Eur J Biochem* **187**: 321–28.
57 Kengen SWM, Luesink EJ, Stams AJM, Zehnder AJB (1993) *Eur J Biochem* **213**: 305–12.
58 Nucci R, Moracci M, Vaccaro C, Vespa N, Rossi M (1993) *Biotechnol Appl Biochem* **17**: 239–50.
59 Leah R, Kigel J, Svendsen I, Mundy J (1995) *J Biol Chem* **270**: 15789–97.
60 Sinnott ML (1990) *Chem Rev* **90**: 1171–202.
61 Trincone A, Pagnotta E (1995) *Biotechnol Lett* **17**: 45–48.
62 Onishi N, Tanaka T (1995) *Appl Environ Microbiol* **61**: 4026–30.
63 Fischer L, Bromann R, Kengen SWM, De Vos WM, Wagner F (1996) *Bio/technology* **14**: 88–91.
64 Nunoura N, Ohdan K, Yano T, Yamamoto K, Kumagai H (1996) *Biosci Biotechnol Biochem* **60**: 188–93.
65 Vieille C, Zeikus JG (1996) *Trends Biotechnol* **14**: 183–90.
66 Volkin DB, Middaugh CR (1992) In *Stability of Protein Pharmaceuticals,* Part A: *Chemical and Physical Pathways of Protein Degradation* (Ahern TJ, Manning MC, eds) pp 215–47, New York: Plenum Press.
67 Ishikawa K, Kimura S, Kanaya S, Morikawa K, Nakamura H (1993) *Protein Eng* **6**: 85–91.

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