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Mutational Analysis of *Thermus caldophilus* GK24 β-Glycosidase: Role of His119 in Substrate Binding and Enzyme Activity

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Three amino acid residues (His119, Glu164, and Glu338) in the active site of *Thermus caldophilus* GK24 β-glycosidase (*Tca* β-glycosidase), a family 1 glycosyl hydrolase, were mutated by site-directed mutagenesis. To verify the key catalytic residues, Glu164 and Glu338 were changed to Gly and Gln, respectively. The E164G mutation resulted in drastic reductions of both β-galactosidase and β-glucosidase activities, and the E338Q mutation caused complete loss of activity, confirming that the two residues are essential for the reaction process of glycosidic linkage hydrolysis. To investigate the role of His119 in substrate binding and enzyme activity, the residue was substituted with Gly. The H119G mutant showed 53-fold reduced activity on 5 mM *p*-nitrophenyl β-D-galactopyranoside, when compared with the wild type; however, both the wild-type and mutant enzymes showed similar activity on 5 mM *p*-nitrophenyl β-D-glucopyranoside at 75°C. Kinetic analysis with *p*-nitrophenyl β-D-galactopyranoside revealed that the k_{cat} value of the H119G mutant was 76.3-fold lower than that of the wild type, but the K_m of the mutant was 15.3-fold higher than that of the wild type owing to the much lower affinity of the mutant. Thus, the catalytic efficiency (k_{cat}/K_m) of the mutant decreased to 0.08% to that of the wild type. The k_{cat} value of the H119G mutant for *p*-nitrophenyl β-D-glucopyranoside was 5.1-fold higher than that of the wild type, but the catalytic efficiency of the mutant was 2.5% of that of the wild type. The H119G mutation gave rise to changes in optima pH (from 5.5–6.5 to 5.5) and temperature (from 90°C to 80–85°C). This difference of temperature optima originated in the decrease of H119G's thermostability. These results indicate that His119 is a crucial residue in β-galactosidase and β-glucosidase activities and also influences the enzyme's substrate binding affinity and thermostability.

Keywords: β-Glycosidase, family 1 glycosyl hydrolase, *Thermus caldophilus* GK24, *Tca* β-glycosidase, site-directed mutagenesis, sequence analysis

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Glycosyl hydrolases catalyze the hydrolysis of glycosidic bonds between carbohydrates, or between a carbohydrate and a noncarbohydrate moiety. These enzymes are shown to be involved in a broad range of biological and biotechnological processes, such as growth and development, host-parasite interactions, signaling events, and biomass conversion [6]. A variety of glycosyl hydrolases have been isolated and characterized from all kinds of living organisms. These glycosyl hydrolases are classified into 106 families based on their amino acid sequence and structural similarities, rather than substrate specificities (updated information is available at http://www.cazy.org/CAZY/fam/acc_GH.html) [10]. Family 1 of the glycosyl hydrolases is a large and important group, and the members of this family are characterized by a high degree of sequence similarity, and a wide range of substrate specificity [2]. This family includes β-glycosidases, β-glucosidases, β-galactosidases, β-mannosidases, 6-phospho-β-glucosidases, 6-phospho-β-galactosidases, and myrosinases. A large number of β-glycosidases are grouped into family 1, and they hydrolyze β-glycosidic linkages as well as catalyze the synthesis of oligosaccharides by reversal of their hydrolytic action [5, 24]. Family 1 enzymes mostly include bacterial, archaeal, plant, and animal glycosidases. Recently, a novel β-glucosidase among family 1 enzymes was isolated from uncultured soil bacteria and characterized [16]. β-Glycosidase and β-glucosidase have many potential applications in various biotechnological processes. For example, the transglycosylation activity of β-glycosidases can be applied in the synthesis of a variety of biologically important compounds [5, 26], such as the conversion of the major ginsenoside Rb1 into the pharmaceutically active minor ginsenoside Rd [16]. In addition, an algal lytic β-glucosidase from *Sinorhizobium kostiense* AFK-13 can be applied in the control of water blooms as an algicidal agent [15].

In our laboratory, a thermostable β-glycosidase, with β-galactosidase, β-glucosidase, and β-fucosidase activities, has been purified from *Thermus caldophilus* GK24 and characterized in detail [34]. This enzyme is active as a monomer and has a molecular mass of 49 kDa. The gene

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             *                 *                 *#                 #                 @     **
Tca  14 TSAYQIEGA 22  115 LTLYHWDLP 123  159 FATLNEPWCS 168  334 LYITENGAA 342  381 GYFVWSLMDNFEWAFGY 397

Bci  16 TAAYQIEGA 24  117 CTLYHWDLP 125  161 WITFNEPWCM 170  351 LYITENGAC 359  398 GYMEWSLMDNFEWAEGY 414
Lla  15 TAAYQAEGA 23  112 VTLHHFDTP 120  155 WTTFNEIGPI 164  371 IYITENGLG 379  417 GYFIWSLMDVFSWSNGY 433
Ppo  16 TAAYQIEGA 24  117 CTLYHWDLP 125  161 WLTFNEPWCI 170  348 IYITENGAC 356  394 GYMAWSLLDNFEWAEGY 410
Ssp  28 TASYQIEGA 36  129 ATLYHWDLP 137  173 WTTLNEPWCS 182  379 LVITENGAA 387  426 GYFLWSLLDNFEWAHGY 442
Tma  16 TASYQIEGS 24  117 VTIYHWDLP 125  161 WITLNEPWVV 170  347 VYITENGAA 355  394 GYFVWSLLDNFEWAEGY 410
Tno  14 TSAYQIEGA 22  115 LTLYHWDLP 123  159 FATLNEPWCS 168  334 LYITENGAA 342  381 GYFVWSLMDNFEWAFGY 397
Tth  14 TSAYQIEGA 22  115 LTLYHWDLP 123  159 FATLNEPWCS 168  334 LYVTENGAA 342  381 GYFVWSLMDNFEWAFGY 397

Pho  15 TSSHQIEGN 23  107 VTLHHFTSP 115  150 VATFNEPMVY 159  320 LYITENGIA 328  358 GYFYWSFMDNYEWKEGF 374
Sso  14 QAGFQSEMG 22  146 LNMYHWPLP 154  201 YSTMNEPNVV 210  383 MYVTENGIA 391  421 GYLHWSLADNYEWASGF 437
Tag  13 SSPFQFEAG 21  147 LNLYHWPLP 155  203 WSTMNEPNVV 212  382 LIVTENGVS 390  420 GYLHWSLTDNYEWAQGF 436

Bbr  15 TASYQIEGG 23  118 VTMYHWDLP 126  162 WITFNEPIAV 171  370 LLITENGYG 378  412 GYTVWSLLDNFEWFYGY 428
Sal  35 SSAYQIEG- 42  137 VTLFHWDLP 145  182 WLTINQLYSV 191  405 IYVTENGIS 413  453 GYLAWALGDNYEFNKGF 469
Sbi  35 TSAYQIEGA 43  139 ITIFHWDTP 147  184 WLTFNEPETF 193  400 MYITENGMG 408  449 GYFAWSLLDNFEWSSGY 465
Tre  29 SSAFQYEGA 37  133 VTLFHWDVP 141  178 WITLNEPWGV 187  393 IYITENGRN 401  442 GYFAWSLFDNMEWDSGY 458
Zma  34 TSAYQIEGA 42  138 VTIFHWDVP 146  186 WLTFNEPQTF 195  402 IYITENGIG 410  453 GYFAWSLLDNFEWFAGF 469
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Fig. 1. Amino acid sequence alignment of the regions containing the active site residues.

The sequence of *Tca* β-glycosidase (*Tca*, AAO15361) is shown compared with those of the following family 1 glycosyl hydrolases: *Bacillus circulans* β-glucosidase (*Bci*, AAA22266), *L. lactis* 6-phospho-β-galactosidase (*Lla*, AAA25183), *P. polymyxa* β-glucosidase (*Ppo*, AAA22263), *Streptomyces* sp. β-glucosidase (*Ssp*, CAA82733), *Thermotoga maritima* β-glucosidase (*Tma*, CAA52276), *T. nonproteolyticus* β-glycosidase (*Tno*, AAF36392), *Thermus thermophilus* β-glycosidase (*Tth*, AAN05439), *P. horikoshii* β-glycosidase (*Pho*, BAA29440), *S. solfataricus* β-glycosidase (*Sso*, AAA72843), *Thermosphaera aggregans* β-glycosidase (*Tag*, AAD43138), *B. brassicae* myrosinase (*Bbr*, AAL25999), *S. alba* myrosinase (*Sal*, P29736), *Sorghum bicolor* β-glucosidase (*Sbi*, AAC49177), *T. repens* β-glucosidase (*Tre*, P26205), and *Z. mays* β-glucosidase (*Zma*, AAA65946). Amino acids that are identical between the *Tca* β-glycosidase and the others are indicated by stippled boxes. #, Key catalytic residues; *, substrate-binding residues; @, residue forming the basal platform.

encoding *T. caldophilus* GK24 β-glycosidase (*Tca* β-glycosidase) has been cloned, sequenced, and overexpressed under the control of the *trp* promoter in *Escherichia coli* [5, 8]. The deduced amino acid sequence of the *Tca* β-glycosidase shows high similarity with those of the family 1 glycosyl hydrolases. The activity and properties of the recombinant enzyme, referred to here as the wild type, were comparable to those of the native enzyme isolated from *T. caldophilus* GK24. The thermostable *Tca* β-glycosidase also has galactosyl transfer activity at high temperatures [5].

The crystal structures of sixteen family 1 glycosyl hydrolases, including *Trifolium repens* β-glucosidase [3], *Lactococcus lactis* 6-phospho-β-galactosidase [33], *Sinapis alba* myrosinase [4], *Sulfolobus solfataricus* β-glycosidase [1], *Paenibacillus polymyxa* β-glucosidase [27], *Thermus nonproteolyticus* β-glycosidase [32], *Pyrococcus horikoshii* β-glycosidase [2], and *Brevicoryne brassicae* myrosinase [12], have been determined. All the structures have essentially the same basic $(\beta/\alpha)_8$ barrel fold. The hydrolysis of glycosidic bonds by family 1 glycosyl hydrolases is done through a double displacement mechanism in which a covalent glycosyl-enzyme intermediate is generated (glycosylation step) and hydrolyzed (deglycosylation step) [7, 21]. Two glutamic acid residues are generally involved in the hydrolytic mechanism: one acting as a nucleophile and the other as a general acid/base catalyst. Along with the two catalytic residues, crystallographic studies have revealed several amino acid residues that are directly involved in forming hydrogen bonds with the glycone hydroxyls of the substrate. The substrate-binding residues are a glutamine, a histidine, an asparagine, a glutamic acid, and a tryptophan, and these residues, like the two catalytic residues, are highly conserved in all family 1 glycosyl hydrolases (refer to Fig. 1). The four residues, except the histidine residue, have been investigated by site-directed mutagenesis and kinetic analysis [9, 13, 20, 28, 31]; all the mutations resulted in large changes in substrate specificity and enzyme activity. The glutamic acid residue, which interacts with glycone hydroxyls 4 and 6, was proposed as a crucial element in determining the preference for galactosides, glucosides, and fucosides [19].

Here, we experimentally identified the putative key catalytic residues Glu164 and Glu338 of *Tca* β-glycosidase by site-directed mutagenesis. We also revealed the role of a putative substrate-binding residue, His119, in the enzyme activity of *Tca* β-glycosidase. To our knowledge, this is the first report of site-directed mutagenesis and kinetic analysis showing the importance of the conserved histidine residue in the substrate binding and enzyme activity of family 1 glycosyl hydrolases.

Materials and Methods

Strain, Plasmid, and Substrates

Escherichia coli W3110 was used as the host for plasmid preparation and gene expression. Plasmid pTRPES [5], containing the *trp* promoter, was used as the cloning and expression vector. The chromogenic substrates *p*-nitrophenyl β-D-galactopyranoside (*p*NPGal) and *p*-nitrophenyl β-D-glucopyranoside (*p*NPGlu) were purchased from Sigma (St. Louis, U.S.A.).

Sequence Analysis

The deduced amino acid sequence of *Tca* β-glycosidase was compared with those of the family 1 glycosyl hydrolases, whose

crystal structures are known. The amino acid sequences were aligned using the ClustalW program based on a Gonnet matrix.

Site-Directed Mutagenesis

Site-directed mutagenesis was performed using the PCR-based overlap extension method [11]. The expression plasmid pTTGL [5], containing the wild-type *Tca* β-glycosidase gene (GenBank Accession No. AF322365) [8], was used as a template in the PCR reactions. The following oligonucleotide primer pairs were synthesized: for the His119Gly mutation, sense primer HGF (5'-CTCACCCTCTAC-***GGC***TGGGACCTGCCCCAG-3') and antisense primer HGR (5'-CAGGTCCCA***GCC***GTAGAGGGTGAGGAAGGGC-3'); for the Glu164Gly mutation, sense primer EGF (5'-GCCACCCTGAAC-***GGG***CCCTGGTGCTCGGCC-3') and antisense primer EGR (5'-GCACCAGGG***CCC***GTTCAGGGTGGCGAAGAA-3'); and for the Glu338Gln mutation, sense primer EQF (5'-CTTTACATCACG-***CAA***AACGGGGCCGCCTAC-3') and antisense primer EQR (5'-GGCGGCCCCGTT***TTG***CGTGATGTAAAGGGGC-3'). The triplets in bold and italics highlight the mutated codons. These primer pairs were used in combination with the 5' (N-terminal) primer (5'-NNNNGAATTCATGACCGAGAACGCCGAAAAG-3', EcoRI site is underlined) and the 3' (C-terminal) primer (5'-NNNNGTCGAC-GCTCAGAGCTGGGCCC-3', SalI site is underlined) [5]. The amplified products were digested with EcoRI and SalI, and were ligated into the expression vector pTRPES [5], which had been digested with the same enzymes, to generate the expression plasmids pTTGLM1 (for the H119G mutant), pTTGLM2 (for the E164G mutant), and pTTGLM3 (for the E338Q mutant). The ligated plasmids were then transformed into *E. coli* W3110, respectively. Clones with the correct construct were selected by restriction enzyme analysis of the plasmid, and introduced mutations were confirmed by nucleotide sequence analysis.

Protein Expression and Purification

E. coli W3110 cells harboring the different expression plasmids (pTTGL, pTTGLM1, pTTGLM2, and pTTGLM3) were cultured in 1 l of M9 minimal medium containing 0.1% (w/v) glucose, 0.5% (w/v) casamino acids, and 100 μg/ml of ampicillin at 37°C for 14 h. The cells were harvested by centrifugation and resuspended in buffer A (10 mM sodium phosphate buffer, pH 6.0) containing 1 mM phenylmethylsulfonyl fluoride. The resuspended cells were disrupted by sonication and centrifuged at 35,000 ×g and 4°C for 20 min to remove the *E. coli* cell walls and insoluble debris. The nucleic acids in the sonicated extracts were precipitated by 1% (w/v) streptomycin sulfate at room temperature for 20 min, and were removed by centrifugation at 35,000 ×g and 4°C for 20 min. The majority of heat-labile *E. coli* proteins were eliminated by a heat treatment at 80°C for 40 min. After removal of the denatured proteins by centrifugation at 35,000 ×g and 4°C for 20 min, the supernatants were dialyzed against buffer A, and were then loaded onto a DEAE-Sephacel column equilibrated with buffer A. The column was washed with ten column volumes of buffer A, and the protein was eluted with a linear gradient of 0–500 mM NaCl prepared in buffer A. The purified proteins were desalted by dialysis against buffer A.

Protein concentration was determined by the method of Lowry *et al.* [18], with bovine serum albumin as a standard. SDS-PAGE was performed, as described by Laemmli [17], with 10% (w/v) polyacrylamide gel.

Enzyme Activity Assay

The β-galactosidase and β-glucosidase activities of the purified enzymes were assayed with 5 mM *p*NPGal and 5 mM *p*NPGlu, respectively, as substrates [34]. The basic reaction mixture contained 0.3 ml of 50 mM sodium phosphate buffer (pH 6.0), 0.2 ml of 15 mM *p*NPGal or *p*NPGlu in 50 mM sodium phosphate buffer (pH 6.0), and 0.1 ml of enzyme solution. This mixture was incubated at 75°C for 5 min, and the reaction was terminated by the addition of 0.4 ml of 0.5 M Na_2CO_3. The extent of hydrolysis was determined from the absorbance of the liberated *p*-nitrophenol at 410 nm, using an extinction coefficient of $1.77\times10^4\ M^{-1}\ cm^{-1}$ [25]. One unit of β-glycosidase is defined as the amount of enzyme that liberated 1 μmol of *p*-nitrophenol at 75°C in 1 min.

To determine the pH dependence of β-glucosidase activity, the reaction was performed in the pH range of 3.5–9.5. To determine the temperature dependence of β-glucosidase activity, the reaction mixture was incubated for 5 min at the indicated temperatures. The thermostability of the enzyme was tested by measuring the decrease in activity after incubation at two different temperatures: 80°C and 90°C.

Kinetic Analysis

The K_m and V_{max} of the wild-type *Tca* β-glycosidase and H119G mutant for the hydrolysis of *p*NPGal (50 mM citrate phosphate buffer, pH 5.5) and *p*NPGlu (50 mM sodium phosphate buffer, pH 6.0) were determined at 75°C. Activity for *p*NPGal was measured at 15 different substrate concentrations (0.5–7.5 mM for the wild type and 1–15 mM for the mutant), and two different amounts of the enzyme (100 ng for wild type and 1 μg for the mutant) were used in the reaction. Activity for *p*NPGlu was measured at 10 different substrate concentrations (0.03–0.3 mM for the wild type and 2–20 mM for the mutant), and 100 ng of enzyme was used in the reaction. The K_m and V_{max} values were calculated from a Lineweaver-Burk plot. The turnover number (k_{cat}) is defined as the amount of substrate molecules converted per second, in which a molecular mass of 49 kDa was used for both the wild-type and mutant enzymes.

RESULTS AND DISCUSSION

Determining the Active Site Residues in *Tca* β-Glycosidase by Sequence Comparison

The deduced amino acid sequence of the *Tca* β-glycosidase was aligned with those of the family 1 glycosyl hydrolases with known crystal structures, from fifteen organisms: seven species from Bacteria, three from Archaea, and five from Eukarya. A high degree of sequence similarity was observed in the regions containing key catalytic residues and substrate-binding residues; sequence alignment also showed that the active-site residues are very well conserved in *Tca* β-glycosidase (Fig. 1). The two motifs TF(L)NEP and I(V)TENG make up the catalytic site, and are involved in catalysis by family 1 glycosyl hydrolases [6, 32]. In particular, the Glu residues (which are conserved in all family 1 glycosyl hydrolases, with the exception of plant myrosinases) in the I(V)TENG and TF(L)NEP motifs are

essential for the catalytic mechanism as the nucleophile and the general acid/base catalyst, respectively [1, 35]. Plant myrosinases lack the general acid/base catalytic residue, because there is no need for protonation assistance in the hydrolysis of their natural substrates [4]. Glu338 and Glu164 in the *Tca* β-glycosidase are the equivalents of the key catalytic residues in other family 1 glycosyl hydrolases, and are therefore proposed as candidates playing the roles of nucleophile and general acid/base catalyst, respectively (Fig. 1). At least five residues are involved in a hydrogen bond network for substrate (glycone moiety) binding: a Gln interacting with glycone hydroxyls 3 and 4, a His with hydroxyl 3 (and/or hydroxyl 2), an Asn with hydroxyl 2, a Glu with hydroxyls 4 and 6, and a Trp with hydroxyl 3 [19, 27]. The first three residues are strictly conserved in the aligned family 1 glycosyl hydrolases, whereas the last two residues are highly conserved. Gln18, His119, Asn163, Glu392, and Trp393 in *Tca* β-glycosidase correspond to the substrate-binding residues in other family 1 glycosyl hydrolases (Fig. 1).

From the above sequence analysis, three residues of the *Tca* β-glycosidase were selected for site-directed mutagenesis. Glu164 and Glu338 were mutated to Gly and Gln, respectively, in order to experimentally prove they are the key catalytic residues of the *Tca* β-glycosidase. His119 was also mutated to Gly in order to reveal the role of this residue in the substrate binding and enzyme activity of the *Tca* β-glycosidase. Among the five substrate-binding residues of the family 1 glycosyl hydrolases, the His was the only one that had not been previously investigated by mutagenesis.

Expression and Purification of the Wild-Type *Tca* β-Glycosidase and Mutants

We obtained three expression plasmids containing individual mutations: His119 (CAC)→Gly (GGC), Glu164 (GAG)→Gly (GGG), and Glu338 (GAA)→Gln (CAA). The wild-type *Tca* β-glycosidase and its mutants were individually expressed in *E. coli* W3110 cells, and purified by heat treatment and DEAE-Sephacel column chromatography. The purification of the enzymes was confirmed by SDS-PAGE. The three mutant enzymes, H119G mutant, E164G mutant, and E338Q mutant, showed the same mobilities as the wild-type enzyme, and their molecular masses were estimated to be 49 kDa (Fig. 2).

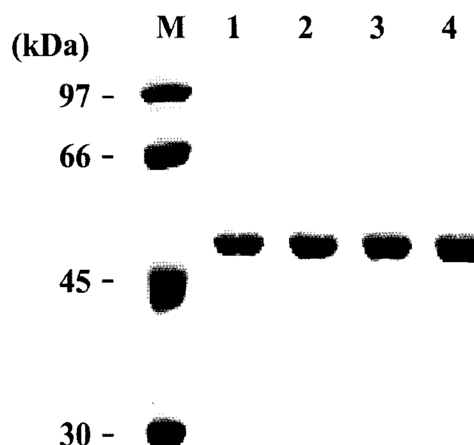

Fig. 2. SDS-PAGE analysis of the purified wild-type and mutant enzymes.

Electrophoresis was performed on a vertical gel of 10% (w/v) polyacrylamide, and the gel that is shown was stained with Coomassie brilliant blue R-250. Lane M, low-molecular-mass markers (molecular masses are indicated at the left); lane 1, wild-type *Tca* β-glycosidase; lane 2, H119G mutant; lane 3, E164G mutant; lane 4, E338Q mutant.

Enzyme Activity Comparison of the Wild-Type *Tca* β-Glycosidase and Mutants

Both the β-galactosidase and β-glucosidase activities of the purified wild-type *Tca* β-glycosidase and its mutants were measured by the hydrolysis of *p*NPGal and *p*NPGlu, respectively, at the final concentration of 5 mM. The specific activities of the wild-type *Tca* β-glycosidase on *p*NPGal and *p*NPGlu (pH 6.5) at 75°C were 83.8 U/mg and 33.2 U/mg, respectively. Both activities of the E164G mutant were dramatically reduced to less than 1% of those of the wild type; the E338Q mutant, although it is a isosteric mutant, was totally inactive against both substrates tested (Fig. 3). These severe losses in enzyme activity for both mutants confirm the importance of Glu164 and Glu338, and are consistent with their presumed roles in the catalytic mechanism. Similar experiments have been done for *S. solfataricus* β-glycosidase [23], *Agrobacterium faecalis* β-glycosidase [29, 31], *Streptomyces* sp. β-glucosidase [30], and *Zea mays* β-glucosidase [35]. Likewise, the mutations of the two key catalytic residues in those enzymes caused drastic reductions in enzyme activity or even complete inactivity. Taken together, the sequence analysis, site-directed mutagenesis, and the comparison with other family 1 glycosyl hydrolases prove that Glu338 and Glu164 are the nucleophile and general acid/base catalyst, respectively, in *Tca* β-glycosidase.

An interesting result was obtained from the H119G mutant. The specific activity (34.1 U/mg) of the H119G mutant on 5 mM *p*NPGlu (pH 5.5) at 75°C was fairly similar to that of the wild type, but the activity (1.6 U/mg) of the H119G mutant on 5 mM *p*NPGal was notably decreased (Fig. 3). To clear this apparent discrepancy, the ability of both enzymes on *p*NPGlu hydrolysis was tested in the different concentration ranges of *p*NPGlu (0.05–10 mM). The specific activity of the wild type was about 3.3 times higher than that of the H119G mutant at a low

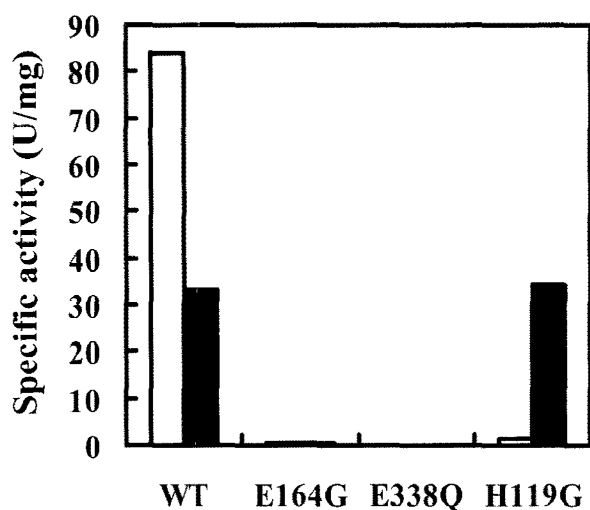

Fig. 3. Enzyme activity comparisons of the wild-type *Tca* β-glycosidase (WT) and its mutants.
The β-galactosidase (open bars) and β-glucosidase (closed bars) activities of the enzymes were assayed with 5 mM *p*NPGal and 5 mM *p*NPGlu, respectively.

concentration such as 0.5 mM *p*NPGlu, but the specific activity of the wild type was similar to that of the H119G mutant at a high concentration such as 5 mM *p*NPGlu (data not shown). At high concentration such as 10 mM *p*NPGlu, the specific activity of the H119G mutant was about 2 times higher than that of the wild type. At low concentration (under 0.5 mM) *p*NPGlu, the specific activity of the H119G mutant (used the same amount as wild type) could not be detected at 75°C for 5 min. This result is in accord with the results that the k_m value of the wild type was very much lower than that of the H119G mutant, but the k_{cat} value of the H119G mutant was higher than that of the wild type, as further described in the kinetic analysis of the H119G mutant. This result indicates that His119 is an important residue for the β-galactosidase activity of the *Tca* β-glycosidase. Considering that the corresponding His residues in other family 1 glycosyl hydrolases are known to be involved in substrate binding [3, 6, 19], the absence of His119 seems to make the binding of the H119G mutant to *p*NPGal difficult, resulting in the very slow hydrolysis of the substrate. However, it is proposed by the β-glucosidase activity of the H119G mutant (comparable to that of the wild type on 5 mM *p*NPGlu) that His119 makes different interactions with each of the β-galactoside and β-glucoside substrates. The effects of His119 on the β-galactoside and β-glucoside substrates were further investigated by the kinetic analysis of the H119G mutant.

Kinetic Analysis of the H119G Mutant

The kinetic parameters of the H119G mutant for *p*NPGal were determined and compared with those of the wild-type *Tca* β-glycosidase. The amount of the H119G mutant used for *p*NPGal was 10-fold (1 μg) compared with that of wild type (100 ng) in the reaction, owing to a very low activity. The K_m and k_{cat} (calculated from V_{max}) values of the H119G mutant were 45.4 mM and 1.9 s^{-1}, respectively (Table 1). The H119G mutant had a 15.3-fold higher K_m, as well as a 76.3-fold lower k_{cat}, as compared with those of the wild type; consequently, the catalytic efficiency (k_{cat}/K_m) of the mutant decreased to 0.08% to that of the wild type. The kinetic parameters of the H119G mutant for *p*NPGlu were determined and compared with those of the wild-type *Tca* β-glycosidase. Owing to a large difference in hydrolysis rate, the reactions were performed in different concentration ranges of *p*NPGlu for the wild-type (0.03–0.3 mM) and mutant (2–20 mM) enzymes. The K_m and k_{cat} (calculated from V_{max}) values of the H119G mutant were 18.14 mM and 154 s^{-1}, respectively (Table 1). The H119G mutant had a 202-fold greater K_m, as well as a 5.1-fold greater k_{cat}, as compared with those of the wild type; consequently, the catalytic efficiency (k_{cat}/K_m) of the mutant decreased to 2.5% to that of the wild type. For reference, the V_{max} of the H119G mutant (18.5 μmol/min/ml) was a 5.2-fold greater than that of the wild type (3.8 μmol/mim/ml). Thus, the specific activity of the wild type was much higher than that of the H119G mutant at a low concentration of *p*NPGlu, but the specific activity of the wild type was similar to that of the H119G mutant at a high concentration such as 5 mM *p*NPGlu (data not shown). The drastic increase in K_m for the H119G mutant indicates that His119 is a critical residue for the affinity of the *Tca* β-glycosidase on the β-galactoside and β-glucoside substrates. Catalytic efficiency decreases have also been shown in all mutants of the other substrate-binding residues of family 1 glycosyl hydrolases, including Trp429 of *L. lactis* 6-phospho-β-galactosidase [28], Asn206 of *Pyrococcus furiosus* β-glucosidase [14], Gln39 and Glu451 of *Spodoptera frugiperda* β-glycosidase [20], and Glu432 and Trp433 of *S. solfataricus* β-glycosidase [9].

Our results show experimentally that His119 is a substrate-binding residue of the *Tca* β-glycosidase, and as expected,

Table 1. Kinetic parameters of the wild-type *Tca* β-glycosidase and H119G mutant with *p*NPGal and *p*NPGlu as a substrate.

	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})	k_{cat}/K_m relative
Wild type for *p*NPGal	2.96±0.5	144.9±20	48.9	100.0
H119G for *p*NPGal	45.4±5	1.9±0.5	0.04	0.08
Wild type for *p*NPGlu	0.09±0.005	30.1±2	334.1	100.0
H119G for *p*NPGlu	18.14±1.87	154±21	8.5	2.5

the mutation of this residue significantly decreases the binding affinity of substrate, and thus, dramatically decreases the enzyme activity and catalytic efficiency.

pH and Temperature Optima and the Thermostability of the H119G Mutant

The effect of pH on the β-glucosidase activity of the H119G mutant was examined in the pH range of 3.5–9.5. The enzyme activity of the mutant was highest at pH 5.5, with a narrow pH range (Fig. 4). In contrast, the wild-type *Tca* β-glycosidase showed optimum activity over a broad pH range, with maximum activity at pH 5.5–6.5. It is known that the pH optimum for the hydrolysis of glycosidic bonds is determined by the ionization constant (pK_a) values of the two key catalytic residues [22]. Since the H119G mutation removes a positive charge in the active site, this might influence the pK_a of the key catalytic residues, and, therefore, shift the pH optimum of the mutant to a lower value. It was also reported by Kaper *et al.* [14] that the introduction or removal of charges in the active site of *P. furiosus* β-glucosidase resulted in significant pH shifts for optimal hydrolysis.

The effect of temperature on the β-glucosidase activity of the H119G mutant was examined in the range of 35–95°C. The temperature optimum of the H119G mutant was 80–85°C, whereas that of the wild type *Tca* β-glycosidase was 90°C (Fig. 5). We supposed that the difference of temperature optima may reflect the some difference of thermostability between the wild-type and H119G mutant. Thus, the thermostabilities of the wild type and H119G mutant were tested by measuring their decrease in activity

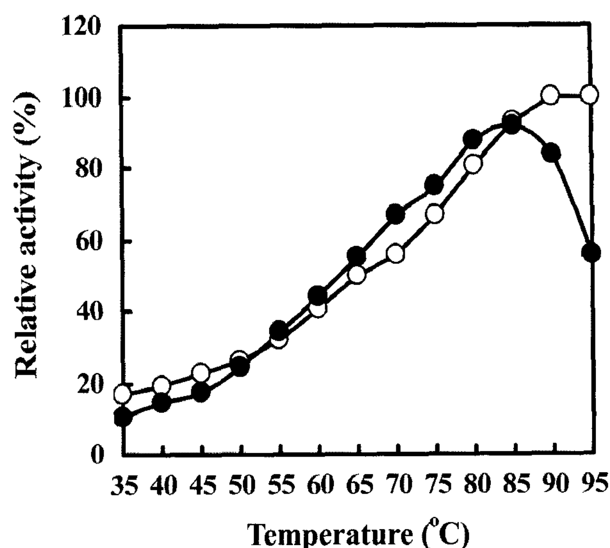

Fig. 5. Effects of temperature on the β-glucosidase activity of the wild-type *Tca* β-glycosidase (○) and H119G mutant (●).
The activity of the wild-type and mutant enzymes was assayed at their optimum pH: 50 mM sodium phosphate buffer (pH 6.5) for the wild type, and 50 mM citrate phosphate buffer (pH 5.5) for the mutant.

after incubation at two different temperatures: 80°C and 90°C. As expected, the β-glucosidase activity of the H119G mutant decreased rapidly as compared with that of the wild type (Fig. 6). Thus, the mutation of His119 brought about the same change of temperature optima.

In conclusion, the role of His119 as a substrate-binding residue was revealed experimentally by site-directed mutagenesis and kinetic analysis. His119 was proven to be a crucial residue for *p*NPGal (β-galactosidase) activity, and

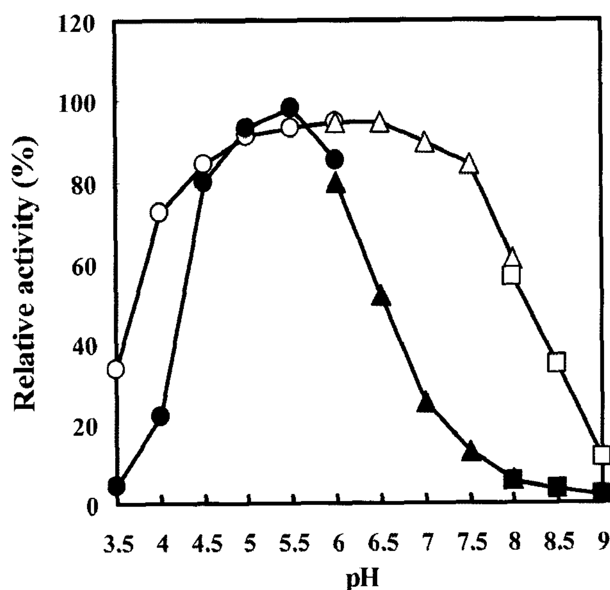

Fig. 4. Effects of pH on the β-glucosidase activity of the wild-type *Tca* β-glycosidase (○, △, □) and H119G mutant (●, ▲, ■): 50 mM citrate phosphate buffer (○, ●), 50 mM sodium phosphate buffer (△, ▲), and 50 mM borax buffer (□, ■).

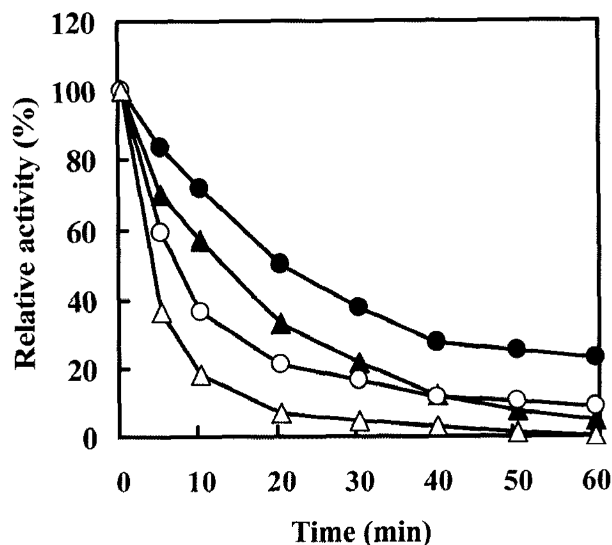

Fig. 6. The thermostability of the β-glucosidase of the wild-type *Tca* β-glycosidase (●, ▲) and H119G mutant (○, △).
The enzymes were incubated at 80°C (○, ●) and 90°C (△, ▲) for an adequate time, and then their residual activities were measured at their optimum pH: 50 mM sodium phosphate buffer (pH 6.5) for the wild type, and 50 mM citrate phosphate buffer (pH 5.5) for the mutant.

the His119G mutation strongly affects the *p*NPGal activity. However, the His119G mutation was not nearly as influential on the apparent *p*NPGlu (β-glucosidase) activity, but showed some changes of catalytic efficiency and themostability.

His119 is strictly conserved in family 1 glycosyl hydrolases from various organisms. This conservation suggests that His119 has the same function in all the family 1 glycosyl hydrolases having β-galactosidase and β-glucosidase activities.

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