Enhancement of the hydrolysis activity of β -galactosidase from *Geobacillus stearothermophilus* by saturation mutagenesis

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ABSTRACT

Thermostable β -galactosidase (BgaB) from *Geobacillus stearothermophilus* is characterized by its thermostability in the hydrolysis of lactose to produce lactose-free milk products. However, BgaB has limited activity toward lactose. We established a method for screening evolved mutants with high hydrolysis activity based on prediction of substrate binding sites. Seven amino acid residues were identified as candidates for substrate binding to galactose. To study the hydrolysis activity of these residues, we constructed mutants by site-saturation mutagenesis of these residue sites, and each variant was screened for its hydrolysis activity. The first round of mutagenesis showed that changes in amino acid residues of Arg109, Tyr272, and Glu351 resulted in altered hydrolysis activity, including greater activity toward ortho-nitrophenyl- β -D-galactopyranoside (oNPG). The mutants R109V and R109L displayed changes in the optimum pH from 7.0 to 6.5, and the mutant R109V/L displayed different substrate affinity and catalytic efficiency (k_{cat}/K_m). Mutant R109G showed complete loss of BgaB enzymatic activity, suggesting that Arg109 plays a significant role in maintaining hydrolysis activity. The optimum pH of mutant E351R increased from 7.0 to 7.5 and this mutant showed a prominent increase in catalytic efficiency with oNPG and lactose as substrates.

Key words: β -galactosidase, *Geobacillus stearothermophilus*, site-saturation mutagenesis, molecular modeling

INTRODUCTION

β -Galactosidase (β -D-galactohydrolase; EC 3.2.1.23, BgaB) is a member of the family of glycosyl hydro-

lases that are known to catalyze the hydrolysis of the β -1,4-D-galactosidic linkage of lactose to its component moiety, such as glucose and galactose (Ly and Withers, 1999). One of the main biotechnological applications of β -galactosidase in the dairy industry is the hydrolysis of lactose from milk for people with lactose intolerance (Kang et al., 2005).

At present, on the basis of amino acid sequence similarity, β -galactosidases are divided into 4 distinct glycoside hydrolase (GH) families, GH-1, GH-2, GH-35, and GH-42 (Henrissat, 1991). They all belong to the superfamily 4/7 with a triose phosphate isomerase (TIM) barrel fold catalytic domain, and the catalytic residues are located at the β -4 and β -7 of the TIM barrel fold. Interestingly, some organisms from which the galactosidases of the GH-42 family originate can survive in extreme circumstances, such as thermophilic, psychrotrophic, halophilic, and acidophilic conditions (<http://www.cazy.org/fam/GH42.html>).

Compared with mesophilic enzymes, thermostable β -galactosidases have significant advantages in the production of low-lactose dairy products (Kengen et al., 1993; Moracci et al., 1995; Petzelbauer et al., 1998; Wolosowska and Synowiecki, 2004). However, experimental data supporting lactose hydrolysis by GH-42 β -galactosidases are absent or weak (Shipkowski and Brenchley, 2006).

Efforts have been made to improve the specific activity and catalytic efficiency of thermostable galactosidases because they have long been considered the critical factors determining the feasibility of use for industrial applications (Hirata et al., 1985; Chen et al., 2008, 2009; Placier et al., 2009). Currently, no structural information is available for BgaB and the only information available is the x-ray crystallography of A4- β -galactosidase (A4- β -Gal) of the GH-42 family. In the present study, a 3-dimensional (3D) structural model of the galactose binding of BgaB was generated by using bioinformatics methods and site-saturation mutagenesis, which was used to identify the key residues and investigate the essential role of amino acids of

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BgaB with respect to substrate binding and biochemical parameters.

MATERIALS AND METHODS

Materials

Oligonucleotides were synthesized and DNA sequencing was carried out at Shanghai Biosune Biotechnology Co. Ltd. (Shanghai, China). The bicinchoninic acid (BCA) protein analysis kit was purchased from Gen-eray Biotech (Shanghai, China). Ortho-nitrophenyl- β -D-galactopyranoside (oNPG), o-nitrophenol, lactose, glucose oxidase, and peroxidase were purchased from Shanghai Sangon Biological Engineering Technology and Services (Shanghai, China).

Bacterial Strains and Growth Conditions

Escherichia coli strain JM109 [*recA1*, *supE44*, *endA1*, *hsdR17*, *gyrA96*, *relA1*, *thi*, Δ [*lac-proAB*] F' (*traD36*, *proAB*⁺, *lacI*^F, *lacZ* Δ M15)] was used as the host for the expression of wild-type and mutant *bgaB* genes. Recombinant *E. coli* strains were routinely grown in Luria-Bertani (LB) medium with 100 μ g/mL of ampicillin.

Construction of the Expression Plasmid

The plasmid pET28a (+)-*bgaB* containing the wild-type *Geobacillus stearothermophilus bgaB* cDNA served as template DNA. The gene-specific primers pKKPri**gaB**_U 5'-CCCGGGGAATTCATGAATGTGTTATCCTCAATT-3' and pKKPri**gaB**_D 5'-AAAAACTGCAGGTGGTGGTGGTGGTGGTGAACCTTCCCGGCTTC-3' were used in this reaction, which introduced an additional *EcoRI* restriction site at the N-terminus *bgaB* sequence and a *PstI* restriction site just behind the stop codon. After digestion with *EcoRI* and *PstI*, the 2,016-bp fragment and a his-tag were inserted into the expression vector pKK223-3 (Pharmacia, Shanghai, China), which was cut with the appropriate restriction enzymes to create the plasmid pKK223-3-*bgaB*.

Construction of Site-Saturation Library

Randomization codon was achieved using an oligonucleotide pair that introduced a codon "NNK" at a selected position (Arnold and Georgiou, 2003). Degenerate primers were employed in a QuikChange PCR reaction (Stratagene, La Jolla, CA) using pKK223-3-*bgaB* as template, with 50 ng of template DNA being combined with 125 ng of each primer, 0.2 mM dNTPs, and 2.5 U of KOD Plus (Toyobo, Japan) in KOD-Plus

buffer. The mixture was subjected to 16 cycles of 30 s at 95°C, 30 s at 55°C, and 6.6 min at 68°C. The reaction products were treated with 20 U of *DpnI* (Fermentas, Glen Burnie, MD) for 1.5 h. Following digestion, high-molecular-weight DNA in the reaction sample was purified from other components using a PCR purification kit (Sangon, Shanghai, China). The resultant plasmid DNA was transformed into 100 μ L of chemically competent JM109 cells. The colonies were pooled and the library DNA was prepared.

Escherichia coli JM109, transformed with site-saturation mutagenized plasmid DNA, was plated on LB agar plates containing 0.1 mg/mL ampicillin. The plates were incubated overnight at 37°C. Individual colonies were randomly selected and *E. coli* JM109 carrying the respective recombinant plasmids were grown in 200- μ L LB cultures in 96-well plates containing ampicillin (100 μ g/mL) overnight at 37°C with agitation (300 rpm). Next, 20 μ L was transferred from each well to a second 96-well plate containing 200 μ L of growth media. The plates were incubated at 37°C with agitation (300 rpm) until the optical density at 600 nm (OD₆₀₀) reached 0.6 to 0.8, at which point the cells were induced with 1 mM isopropyl thio- β -D-galactoside (IPTG). The cells were harvested 24 h after induction by centrifugation at $1,157 \times g$. The cell pellets were resuspended in 200 μ L of 50 mM potassium phosphate buffer (pH 7.0) containing lysozyme (0.5 mg/mL) and incubated on a shaker for 30 min at room temperature. The whole-cell lysates were used as the enzyme source to screen the activity.

Screening Procedure

The screening assay was an adaptation of the microplate reader assay for β -glycosidase activity (Petzelbauer et al., 1998). The mixture contained 200 μ L of saturated oNPG solution (22 mM) in 50 mM potassium phosphate buffer, pH 7.0. The reaction was started with the addition of 20 μ L of lysate. The absorbance at 405 nm was measured on a SpectraMax M5 (Molecular Devices, Sunnyvale, CA) before addition of the crude extract and after incubation at 55°C for 10 min. After analysis of the activity data, plasmid DNA of selected stocks was extracted and sequenced to identify the codon at the randomized position.

Enzyme Expression and Purification

Each protein was fused to a C-terminal 6 histidine-tag. After sequence analysis, the selected mutants and wild-type enzyme stocks were grown in LB medium. When the cell density reached an OD₆₀₀ of 0.6 to 0.8, IPTG was added to the culture to a final concentration of 1 mM. The cultures were further grown at 30°C with

Figure 1. Predicted 3-dimensional structure of thermostable β -galactosidase (BgaB) in GH-42 from *Geobacillus stearothermophilus*. Homology model generated with Program Modeler based on the 3-dimensional structure of A4- β -Gal (Protein Data Bank code: 1KWG). The protein is represented by a ribbon model prepared using a stereo view of BgaB, with the elements of secondary structure shown in cartoon representation: α -helices are shown as ribbons (purple), β -strands are shown as arrows (yellow), and loops as threads (gray). The main structure of the homology model of BgaB is primarily composed of a triose phosphate isomerase (TIM) barrel domain that consists of an inner core of 8 parallel β -strands surrounded by 8 α -helices. Color version available in the online PDF.

shaking for 20 h. The cells were harvested by centrifugation, washed, and resuspended in 50 mM potassium phosphate at buffer pH 7.0. The proteins of small cultures were extracted by sonication. The recombinant enzymes were purified in a 2-step procedure: heat-denaturation of *E. coli* cell proteins at 55°C for 10 min and immobilized metal-ion affinity chromatography. The crude cell-free extracts were obtained from the supernatant following centrifugation at $12,000 \times g$ at 4°C for 15 min. Extracts were purified to homogeneity using nickel chelation affinity chromatography and eluted in buffer containing 250 mM imidazole. Homogeneous fractions were pooled, and imidazole was removed by the Amicon Ultra Centrifugal Filters Device (Millipore, Billerica, MA) with a 30-kDa cut-off membrane. Protein preparations were evaluated by SDS-PAGE and Coomassie Blue staining. The protein concentration was measured using the BCA assay using BSA as standard.

Assay of Galactosidase Activity

The activities of BgaB variants were determined as described by Gist-Brocades (Stellmach, 1988); the incubation temperature of the enzyme was adjusted to

55°C (Xia et al., 2010). One unit of enzyme activity was defined as the amount of enzyme hydrolyzing 1 μ mol of substrate (oNPG) per minute. The specific activity was expressed as units per milligram of cellular or enzyme protein.

Enzymatic hydrolysis of lactose was carried out in a standard lactase assay described previously (Petzelbauer et al., 1998). The incubation temperature of the reaction mixture was decreased from 80°C to 55°C. One unit of lactase activity refers to 1 μ mol of D-glucose released per minute.

The pH versus activity profiles (5.0–9.0) were determined at 55°C in 2 buffer systems: sodium citrate-phosphate buffer (200 mM) from pH 5.0 to 7.5 and sodium potassium-phosphate buffer (200 mM) from pH 7.5 to 9.0.

Kinetic properties were determined with respect to varying concentrations of the chromogenic substrate oNPG (0.1–15 mM) and the natural substrate lactose (0–200 mM). The kinetic parameters were determined by analysis of the Lineweaver-Burk curves.

Amino Acid Sequence Analyses and Homology Modeling

Homologs of BgaB were searched in the nonredundant National Center for Biotechnology Information database using PSI-BLAST (Johnson et al., 2008). Multiple sequence alignment was carried out using the program ClustalX (version 1.81; Thompson et al., 1997) with the default parameters. To highlight conserved residues, the alignment from ClustalX was submitted to ESPript (Gouet et al., 2003).

The 3D structure of BgaB was built using Program Modeler (version 9v4; <http://salilab.org/modeller/>) which enables the user to run an automated approach of comparative modeling by satisfaction of spatial restraints with standard parameters (Karplus and Sali, 1995; Sali et al., 1995). To understand the mechanisms of ligand binding and the interaction between substrate and β -galactosidase, a 3D model of BgaB complexed with galactose was produced using PathDock (Duhovny et al., 2002; Schneidman-Duhovny et al., 2005). The final structure was validated with Procheck (Kengen et al., 1993) and the energetic architecture of the protein folds was determined using ProSA (Wiederstein and Sippl, 2007). Ligplot (Wallace et al., 1995) was used to study the protein–ligand interactions.

RESULTS AND DISCUSSION

Selection of Residues for Mutagenesis

Using the structure of β -galactosidase bound to galactose (Protein Data Bank code 1KWG; <http://>

www.pdb.org/pdb/home/home.do) as a guide, the 3D structure of BgaB was predicted by molecular modeling (Figure 1). The 3D model showed a canonical TIM barrel consisting of an inner core of 8 parallel β -strands surrounded by 8 α -helices (Figure 2). The catalytic residues of BgaB, Glu148 and Glu303, are located at the C-terminal ends of β -4 and β -7 of the TIM barrel, respectively (Figure 2), which is consistent with the characteristics of β -galactosidases belonging

to the superfamily 4/7 (Juers et al., 1999; Nagano et al., 2001). We found that residues Arg109, Asn147, Glu148, Glu303, Tyr272, Trp311, Phe341, Glu351, and His354 participated in interactions with the galactose (Figure 3) as hydrogen bonds between the enzyme and substrate are crucial in the hydrolysis reaction. Seven residues, excluding the catalytic residues E148 and E303, were considered to be suitable candidates for mutagenesis.

Figure 2. Primary structure alignment of β -galactosidase (BgaB No. P19668) and A4- β -galactosidase in GH-42 family from *Thermus thermophilus* A4 (A4- β -Gal No. O69315). The amino acid residues of BgaB identified as substrate-binding sites in this study are indicated by black triangles. Strongly conserved regions are shown in highlight boxes and identical conserved regions in white boxes. The secondary structure of A4- β -Gal is depicted with α -helices in squiggles and β -strands in arrows. Color version available in the online PDF.

Site-Saturation Mutagenesis and Screening

Randomization at the chosen positions was carried out as described in the Materials and Methods, using QuikChange PCR (Stratagene) with degenerate primers. Details of the primers used in the construction of the library are provided in Table 1.

In the initial round of site-saturation mutagenesis, the 7 libraries corresponding to individual positions were screened in 96-well plates. Of the 7 positions randomized in the first round, 4 did not affect activity when oNPG was used as substrate (data not shown). The other 3 libraries, however, showed slightly increased hydrolysis activity; the screening results are shown in Figure 4. In library R109X, most substitutions at position 109 dramatically reduced the hydrolysis activity. However, several colonies showed apparently increased OD₄₀₅ absorbance. Sequencing of the plasmid DNA isolated from these colonies with increased hydrolysis activity showed that Arg109 had been replaced by tryptophan, valine, or leucine.

The absorbance obtained from the colonies produced with the Y272X and E351X libraries did not alter dramatically, indicating that mutations in positions Y272 and E351 may not affect the hydrolysis activity of BgaB as strongly as that of R109. Several colonies showed increases in activity toward oNPG. We selected those colonies that had an absorbance greater than or similar to that of the wild-type; sequencing revealed that these colonies were Y272L, Y272A, Y272S, and E351R.

Purification and Assay for Activity of BgaB Mutants

The wild-type and mutant enzymes R109L, R109V, R109W, Y272L, Y272S, Y272A, and E351R were purified. Using oNPG as substrate, the specific activities of all mutants were determined and compared with that of the wild-type enzyme (Table 2). The results indicated that the specific activity of R109L, Y272L, and R109V was lower and that of Y272S, Y272A, and E351R was higher than the specific activity of the wild-type. Mutant E351R had the highest specific activity,

Figure 3. A drawing of the surrounding amino acid residues interacting with the galactose molecule. Hydrogen bonds are shown as dotted (green) lines. Residues Arg109, Asn147, Glu148, Glu303, Tyr272, Trp311, Phe 341, Glu351, and His354 participate in the interactions with galactose. Color version available in the online PDF.

Table 1. Nucleotide sequences of primers used in saturation mutagenesis of the β -galactosidase (BgaB) gene, *bgab*

Primer	Positions of primers at <i>bgab</i> gene	Sequence ¹
R109X	301–351	5'-CGGTGTCATTCTCTCGTTTGGCAGTNNKCAACATTATTGTCCTAATCATCC-3'
N147X	414–466	5'-GGCACTCAAAATGTGGCATGTTAATNNKGAGTATGCATGTCACGTTTCCAAGT-3'
Y272X	789–841	5'-TGTAGATATTGTGACATGGGACTCANNKCCCTGATCCCAGAGAGGGCTTGCCAA-3'
W311X	906–958	5'-GGAGCAGGTAACCTCACATGTTAACNNKCGCGATATTAATGTTCCAAAACCGC-3'
F341X	996–1048	5'-CCGTGGTGCGATGGTATTATGTTTNNKCAGTGGCGTCAAAGTAGAGCAGGAG-3'
E351X	1026–1078	5'-GTGGCGTCAAAGTAGAGCAGGAGCTNNKAAATTCCACGGTGCAATGGTGCCCC-3'
H354X	1035–1087	5'-AAGTAGAGCAGGAGCTGAAAAATTNNKGGTGCAATGGTGCCCCACTTTTTGA-3'

¹N = equal mixture of A, T, G, and C; K = equal mixture of T and G.

approximately 3.5-fold higher than that of the wild-type enzyme.

The mutant and the wild-type enzymes expressed in recombinant *E. coli* were isolated and purified to homogeneity. As shown by the single protein band on SDS-PAGE, the apparent molecular mass of these highly purified enzymes was 70 kDa, as expected for BgaB (Figure 5).

pH Dependency of the Mutant Enzymes

The pH dependency of BgaB and the 7 mutants is shown in Figure 6A. The mutant enzymes R109L and R109V demonstrated a significant change in their optimum pH from 7.0 for the wild-type enzyme to 6.5, and the mutant enzymes Y272L and Y272A (Figure 6B) displayed a similar behavior leading to a decrease in the

Figure 4. Activity plots of 88 colonies obtained from a library of site-saturation mutants of (A) Y272X, (B) E351X, and (C) R109X. The activity of whole cell extract was measured as described in Materials and Methods. In each case, the first 8 bars are the controls of wild-type enzyme (black). mAU = milliabsorbance units.

Figure 5. Sodium dodecyl sulfate-PAGE analysis of the purified wild-type and mutant proteins of β -galactosidase (BgaB). Lanes 1–8: R109L, R109W, R109V, wild-type BgaB, Y272S, Y272L, Y272A, and E351R, respectively; lane M = molecular marker.

optimum pH. The optimum pH of R109W and Y272S increased slightly (Figure 6A), whereas that of E351R (Figure 6B) increased to 7.5 compared with that of the wild-type enzyme.

The substitution of Arg109, which is located in a loop between β -strand 3 and α -sheet 3 (Figure 2), can be inferred to induce a change in the optimal pH of the enzymes. In addition, Arg109 is involved in the binding of O3 and O4 of the galactose molecule by direct hydrogen bond interactions (Figure 3). The substitution at this position may be implicated in substrate recognition because the mutated residues at these sites may disturb the hydrogen bond network and change the pH optimum of the mutant enzymes compared with that of the wild-type enzyme. Placier et al. (2009) obtained the evolved mutant enzymes of BgaB after the random mutagenesis approach, and the mutant carrying Lys instead of Arg at position 109 may be responsible for the increased transglycosylation capacity. The difference between glycosidases and glycosyl transferases depends on the acceptor. For glycosyl transferases the acceptor is glycoside, whereas for glycosidases the ac-

Figure 6. Effect of pH on the activity of purified β -galactosidase (BgaB) wild-type and mutant enzymes: (A) wild-type (WT), R109W, R109V, and R109L; (B) WT, Y272L, Y272A, Y272S, and E351R. The pH profiles were determined in different buffers by varying pH values from 5.0 to 9.0 at 55°C.

ceptor is water, the result being hydrolysis (Ly and Withers, 1999). Consistent with our findings, Placier et al. (2009) also demonstrated that Arg109 may play an important role in substrate recognition.

Characterization of Variants

Enzyme kinetics with oNPG and lactose as substrates were compared (Table 3). All selected vari-

Table 2. Specific activities of the wild-type and mutant β -galactosidase (BgaB) enzymes using ortho-nitrophenyl- β -D-galactopyranoside (oNPG) as substrate

Enzyme	Specific activity (units/mg of protein)	Specific activity relative to wild-type (%)
Wild-type BgaB	1.86 $\pm$ 0.13	100 $\pm$ 3.6
R109L	1.47 $\pm$ 0.18	79 $\pm$ 3.5
Y272L	1.60 $\pm$ 0.11	86 $\pm$ 1.8
R109V	1.94 $\pm$ 0.19	104 $\pm$ 2.9
R109W	2.27 $\pm$ 0.21	122 $\pm$ 2.5
Y272S	2.52 $\pm$ 0.12	135 $\pm$ 3.1
Y272A	3.26 $\pm$ 0.25	175 $\pm$ 1.1
E351R	6.56 $\pm$ 0.23	353 $\pm$ 3.9

Table 3. Kinetic parameters¹ of purified wild-type and mutant β -galactosidase (BgaB) enzymes for hydrolysis of ortho-nitrophenyl- β -D-galactopyranoside (oNPG) and lactose

Enzyme	oNPG				Lactose			
	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ ·mM ⁻¹)	Relative k_{cat}/K_m (%)	K_m (mM)	k_{cat} (s ⁻¹)	k_{cat}/K_m (s ⁻¹ ·mM ⁻¹)	Relative k_{cat}/K_m (%)
Wild-type BgaB	2.9 ± 0.6	7.6 ± 0.5	2.62	100	110 ± 5.9	0.43 ± 0.13	3.91	100
R109L	14.2 ± 0.4	2.3 ± 0.3	0.16	6	135 ± 7.6	0.21 ± 0.11	1.56	40
R109V	9.1 ± 0.7	5.8 ± 0.4	0.64	24	127 ± 6.1	0.24 ± 0.06	1.89	48
Y272L	6.4 ± 0.5	13.8 ± 0.6	2.16	82	113 ± 3.2	0.46 ± 0.12	4.69	42
R109W	8.9 ± 0.6	40.1 ± 1.2	4.51	172	104 ± 5.1	0.53 ± 0.17	5.10	130
Y272S	5.6 ± 0.3	19.2 ± 0.8	3.43	131	138 ± 3.7	2.18 ± 0.14	15.80	404
Y272A	3.5 ± 0.4	15.1 ± 1.3	4.31	164	106 ± 6.7	0.68 ± 0.15	6.42	164
E351R	3.9 ± 0.3	45.8 ± 1.1	11.74	448	142 ± 9.2	3.86 ± 0.19	27.89	713

¹ K_m = Michaelis constant; k_{cat} = turnover rate; k_{cat}/K_m = catalytic efficiency.

ants displayed an increase in Michaelis constant (K_m) toward oNPG compared with the wild-type enzyme. The mutant enzymes R109V and R109L displayed 24% and 6% decreases in their catalytic efficiency (k_{cat}/K_m , where k_{cat} = turnover rate) compared with the wild-type enzyme. This decrease in catalytic efficiency could be attributed to the interaction of the Arg109 with the substrate, thus inducing a decrease in the substrate affinity and catalytic efficiency. The most considerable increase in catalytic efficiency was observed with the mutant enzymes E351R and R109W, which were approximately 4- and 2-fold more efficient, respectively, than the wild-type enzyme. The turnover rate (k_{cat}) increased for the hydrolysis of oNPG, resulting in increases in catalytic efficiency toward oNPG (Table 3). Again, these results demonstrate the essential role of Arg109 in the kinetic behavior of the *G. stearothermophilus* β -galactosidase.

In agreement with previous reports (Hirata et al., 1985), wild-type BgaB exhibited a low turnover rate and lactose binding affinity, resulting in weak catalytic efficiency (k_{cat}/K_m) toward lactose hydrolysis (Table 3). The catalytic efficiencies of the variants compared with that of the wild-type showed greater values of k_{cat}/K_m for oNPG and lesser values for lactose.

The relative catalytic efficiency for lactose hydrolysis was improved by approximately 4- and 7-fold in mutants Y272S and E351R, respectively. Although the mutants Y272S and E351R displayed an increase in K_m toward lactose compared with the wild-type, the relative turnover rate increased for lactose hydrolysis, resulting in an increase in the relative k_{cat}/K_m toward lactose (Table 3).

To further study the role of Arg109 in enzyme hydrolysis activity, saturation mutagenesis at Arg109 position was carried out. The effects of the mutations on enzyme hydrolysis activity were determined (data not shown). Surprisingly, we found that hydrolysis activity was completely abolished when Arg109 was replaced

with glycine, which suggested that Arg109 might play a significant role in maintaining enzyme activity. An alternative possibility would be that mutation of Arg109 to glycine may cause the loss of ability of substrate recognition.

CONCLUSIONS

Using a combined site-saturation mutagenesis and 3D molecular modeling approach, the current paper presents for the first time a detailed structure–function relationship study of BgaB from the GH-42 family. We established a method for screening of evolved mutants with high hydrolysis activity based on prediction of substrate binding sites and subsequently constructed mutants by saturation mutagenesis of these binding sites. We successfully identified several amino acid mutations that affect the enzyme hydrolysis activity of BgaB. The findings of this work corroborated the crucial effects of residue Arg109 on enzymatic behavior. In addition, this work allowed the generation of some efficient mutant enzymes; the single mutations of R109W, E351R, and Y272S resulted in increased enzymatic activity. The findings presented in the current study could be further applied to the modification of other β -galactosidases of the GH-42 family.

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REFERENCES

- Arnold, F. H., and G. Georgiou, 2003. Methods in Molecular Biology (Directed Evolution Library Creation Methods and Protocols). Humana Press Inc., Totowa, NJ.

- Chen, W., H. Chen, Y. Xia, J. Yang, J. Zhao, F. Tian, H. P. Zhang, and H. Zhang. 2009. Immobilization of recombinant thermostable beta-galactosidase from *Bacillus stearothermophilus* for lactose hydrolysis in milk. *J. Dairy Sci.* 92:491–498.
- Chen, W., H. Chen, Y. Xia, J. Zhao, F. Tian, and H. Zhang. 2008. Production, purification, and characterization of a potential thermostable galactosidase for milk lactose hydrolysis from *Bacillus stearothermophilus*. *J. Dairy Sci.* 91:1751–1758.
- Duhovny, D., R. Nussinov, and H. J. Wolfson. 2002. Efficient unbound docking of rigid molecules. *Lect. Notes Comput. Sci.* 2452:185–200.
- Gouet, P., X. Robert, and E. Courcelle. 2003. ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins. *Nucleic Acids Res.* 31:3320–3323.
- Henrissat, B. 1991. Sequence homology between a beta-galactosidase and some beta-glucosidases. *Protein Seq. Data Anal.* 4:61–62.
- Hirata, H., S. Negoro, and H. Okada. 1985. High production of thermostable beta-galactosidase of *Bacillus stearothermophilus* in *Bacillus subtilis*. *Appl. Environ. Microbiol.* 49:1547–1549.
- Johnson, M., I. Zaretskaya, Y. Raytselis, Y. Merezuk, S. McGinnis, and T. L. Madden. 2008. NCBI BLAST: A better web interface. *Nucleic Acids Res.* 36(Web Server issue):W5–9.
- Juergens, D. H., R. E. Huber, and B. W. Matthews. 1999. Structural comparisons of TIM barrel proteins suggest functional and evolutionary relationships between beta-galactosidase and other glycohydrolases. *Protein Sci.* 8:122–136.
- Kang, S. K., K. K. Cho, J. K. Ahn, J. D. Bok, S. H. Kang, J. H. Woo, H. G. Lee, S. K. You, and Y. J. Choi. 2005. Three forms of thermostable lactose-hydrolase from *Thermus* sp. IB-21: Cloning, expression, and enzyme characterization. *J. Biotechnol.* 116:337–346.
- Karplus, M., and A. Sali. 1995. Theoretical studies of protein folding and unfolding. *Curr. Opin. Struct. Biol.* 5:58–73.
- Kengen, S. W., E. J. Luesink, A. J. Stams, and A. J. Zehnder. 1993. Purification and characterization of an extremely thermostable beta-glucosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. *Eur. J. Biochem.* 213:305–312.
- Ly, H. D., and S. G. Withers. 1999. Mutagenesis of glycosidases. *Annu. Rev. Biochem.* 68:487–522.
- Moracci, M., R. Nucci, F. Febbraio, C. Vaccaro, N. Vespa, F. La Cara, and M. Rossi. 1995. Expression and extensive characterization of a beta-glycosidase from the extreme thermoacidophilic archaeon *Sulfolobus solfataricus* in *Escherichia coli*: Authenticity of the recombinant enzyme. *Enzyme Microb. Technol.* 17:992–997.
- Nagano, N., C. T. Porter, and J. M. Thornton. 2001. The (betaalpha)(8) glycosidases: Sequence and structure analyses suggest distant evolutionary relationships. *Protein Eng.* 14:845–855.
- Petzelbauer, I., B. Nidetzky, D. Haltrich, and K. D. Kulbe. 1998. Development of an ultra-high-temperature process for the enzymatic hydrolysis of lactose. I. The properties of two thermostable beta-glycosidases. *Biotechnol. Bioeng.* 64:322–332.
- Placier, G., H. Watzlawick, C. Rabiller, and R. Mattes. 2009. Evolved beta-galactosidases from *Geobacillus stearothermophilus* with improved transgalactosylation yield for galacto-oligosaccharide production. *Appl. Environ. Microbiol.* 75:6312–6321.
- Sali, A., L. Pottorff, F. Yuan, H. van Vlijmen, and M. Karplus. 1995. Evaluation of comparative protein modeling by MODELLER. *Proteins* 23:318–326.
- Schneidman-Duhovny, D., Y. Inbar, R. Nussinov, and H. J. Wolfson. 2005. PatchDock and SymmDock: Servers for rigid and symmetric docking. *Nucleic Acids Res.* 33(Web Server issue):W363–367.
- Shipkowski, S., and J. E. Brenchley. 2006. Bioinformatic, genetic, and biochemical evidence that some glycoside hydrolase family 42 beta-galactosidases are arabinogalactan type I oligomer hydrolases. *Appl. Environ. Microbiol.* 72:7730–7738.
- Stellmach, B. 1988. Bestimmungsmethoden Enzyme. Steinkopff Verlag, Darmstadt, Germany.
- Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 25:4876–4882.
- Wallace, A. C., R. A. Laskowski, and J. M. Thornton. 1995. LIGPLOT: A program to generate schematic diagrams of protein-ligand interactions. *Protein Eng.* 8:127–134.
- Wiederstein, M., and M. J. Sippl. 2007. ProSA-web: Interactive web service for the recognition of errors in three-dimensional structures of proteins. *Nucleic Acids Res.* 35(Web Server issue):W407–410.
- Wolosowska, S., and J. Synowiecki. 2004. Thermostable beta-glucosidase with a broad substrate specificity suitable for processing of lactose-containing products. *Food Chem.* 85:181–187.
- Xia, Y., J. Zhao, H. Chen, X. Liu, Y. Wang, F. Tian, H. P. Zhang, H. Zhang, and W. Chen. 2010. Extracellular secretion in *Bacillus subtilis* of a cytoplasmic thermostable β -galactosidase from *Geobacillus stearothermophilus*. *J. Dairy Sci.* 93:2838–2845.