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Biochemical characterization of mutants in the active site residues of the β-galactosidase enzyme of *Bacillus circulans* ATCC 31382

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ABSTRACT

The *Bacillus circulans* ATCC 31382 β-galactosidase (BgaD) is a retaining-type glycosidase of glycoside hydrolase family 2 (GH2). Its commercial enzyme preparation, Biolacta N5, is used for commercial-scale production of galacto-oligosaccharides (GOS). The BgaD active site and catalytic amino acid residues have not been studied. Using bioinformatic routines we identified two putative catalytic glutamates and two highly conserved active site histidines. The site-directed mutants E447N, E532Q, and H345F, H379F had lost (almost) all catalytic activity. This confirmed their essential role in catalysis, as general acid/base catalyst (E447) and nucleophile (E532), and as transition state stabilizers (H345, H379), respectively.

1. Introduction

The β-galactosidase of *Bacillus circulans* ATCC 31382 (BgaD) belongs to the family 2 of glycoside hydrolases (GH2) [1,2]; its physiological role is to hydrolyze lactose into galactose and glucose. Its commercial preparation Biolacta N5 has 4 C-terminally truncated active isoforms, products of proteolysis. Recently, we have cloned the *B. circulans bgaD* gene and characterized the four similar recombinant isoforms rBgaD-(A-D) and the four Biolacta N5 purified isoforms β-GalA-D. The results showed that the smallest isoforms β-Gal-D and rBgaD-D have the same GOS product profiles as the longer and full-length proteins [3]. The GH2 family members have an active site which is composed of two catalytic residues: a catalytic nucleophile and a general acid/base catalyst. GH2 family enzymes are β-retaining and employ a double displacement mechanism (Fig. 1). First, the catalytic nucleophile attacks the anomeric center of the sugar, generating the galactosyl–enzyme intermediate. Subsequently, this intermediate undergoes hydrolysis or transglycosylation. Both steps require assistance of a general acid/base catalyst [4]. At high lactose concentrations some β-galactosidases switch from a hydrolytic to a synthetic mechanism whereby lactose (instead of water) acts as acceptor for the covalently bound galactose [5]. The newly formed product is a galacto-oligosaccharide (GOS).

The *B. circulans* β-galactosidase (monomeric protein, 1737 amino acids, metal independent, catalytic residues unknown [6]) has high transgalactosylation activity and is applied at industrial scale to produce GOS [6,7]. The enzyme shares 22% sequence identity with the *Escherichia coli* β-galactosidase (tetrahomomeric protein, 1024 amino acids, metal-ion dependent, 2 Glu catalytic residues) which has been well characterized [8–15]. Whereas the *B. circulans* β-galactosidase activity is metal independent [6], the *E. coli* β-galactosidase requires both Mg^{2+} and Na^{+} for maximal activity: the sodium ion directly ligands the lactose 6-hydroxyl and the magnesium ion may contribute to the role of the general acid/base catalyst [16]. In contrast to *B. circulans* β-galactosidase, the *E. coli* enzyme is a poor producer of GOS [16]. In the *E. coli* β-galactosidase enzyme the amino acid residues E461 and E537 are respectively the catalytic proton donor and the nucleophile [14]. Moreover, residue H358 was identified as a transition state stabilizer following the observation that it forms strong hydrogen bonds with the C3′ hydroxyl group of the galactosyl unit [10]. Another study showed that the nitrogen of residue H392 is within H-bonding distance of the C3′ hydroxyl group of the galactosyl unit and thereby promotes

Abbreviations: DNA, deoxyribonucleic acid; GOS, galacto-oligosaccharides; GH2, glycoside hydrolase family 2; T_m, melting temperatures; o-NPG, ortho-nitrophenyl-β-galactoside; PCR, polymerase chain reaction; SD, standard deviation

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catalysis by stabilizing the transition state [9]. Here we characterized site-directed mutants of the two putative catalytic glutamates in *B. circulans* β-galactosidase and two conserved histidines in the active site. This is the first report on mutagenesis of the putative Glu catalytic residues of *B. circulans* β-galactosidase, showing that a change in these residues results in loss of β-galactosidase activity.

2. Materials and methods

2.1. General

B. circulans ATCC 31382, obtained from LGC Standards (Wesel, Germany), was grown at 30 °C in 3% (w/v) beef extract and 5% (w/v) peptone. *E. coli* strain TOP10F (Invitrogen, Carlsbad, CA) was used for cloning purposes and grown at 37 °C in Luria–Bertani medium. For plasmid selection the appropriate antibiotic was added at the following concentrations: 50 μg ml^{-1} for kanamycin and 100 μg ml^{-1} for ampicillin. *E. coli* BL21 (DE3) grown at 30 °C was used for high-level expression of β-galactosidases of strain ATCC 31382. Plasmids pZErO-2 and pET15b (both Invitrogen) were used for cloning and expression purposes, respectively. Protein concentrations were determined using the Bradford reagent (Bio-Rad, Munich, Germany) with bovine serum albumin as standard.

2.2. DNA preparation and manipulation

Genomic DNA of *B. circulans* ATCC 31382 was isolated from an overnight grown nutrient broth culture using the GenElute Bacterial Genomic DNA kit (Sigma) according to the recommendations of the manufacturer. Preparation of plasmid DNA cultures of *E. coli* was performed using the GenElute Plasmid Miniprep Kit (Sigma) according to the recommendations of the manufacturer. DNA restriction, ligations, and transformation were performed according to protocols described by Sambrook et al. [17].

2.3. PCR amplification

PCR was performed with genomic DNA of *B. circulans* ATCC 31382 as template to prepare the DNA fragment that correspond to isoform β-Gal-D as found in Biolacta N5 using the common sense primer supplemented by the *Nco*I restriction enzyme site (5′-TATACCATGG GAAACAGTGTGAGCTATGATGG-3′) and anti-sense primer by the *Bgl*II site (rBgaD-D, 5′-TTTAGATCTTTATGGCGTTACACGTAAATAC-3′). The obtained PCR product was purified using the GenElute PCR Clean-Up Kit (Sigma), and blunt ligated into the *Eco*RV site of pZErO-2 followed by a digestion with *Nco*I and *Bgl*II and separated with agarose gel electrophoresis. DNA fragment with the right size was cut out, purified with the GenElute Gel Extraction Kit (Sigma) and ligated into pET-15b to prepare expression plasmid pET-15b-rBgaD-D.

2.4. Bioinformatic analyses

Using T-Coffee [3,18], the *B. circulans* rBgaD-D amino acid sequence was aligned with seven other family GH2 β-galactosidases (Table 1). This alignment was improved manually with Jalview 2.8 [19] by careful examination with Pymol of a 3D BgaD (a.a. 50–650) model queried using PHYRE [20] which was structurally aligned with crystal structures of *Kluyveromyces lactis* BgaL, *E. coli* LacZ and *Arthrobacter* sp. C2-3 LacZ.

2.5. Site-directed mutagenesis

Site-directed mutagenesis of the *B. circulans rbgaD-D* gene was carried out with the QuikChange™ site directed mutagenesis kit (Stratagene) and confirmed by sequencing. *Pfu* Turbo DNA polymerase (Stratagene) was used for all polymerase chain reactions (PCR) (95 °C, 30 s; 55 °C, 1 min; 68 °C, 10 min for 30 cycles) using plasmid pET15b-rBgaD-D as template [3]. PCR products were digested with *Dpn*I to specifically digest the DNA templates. Subsequently,

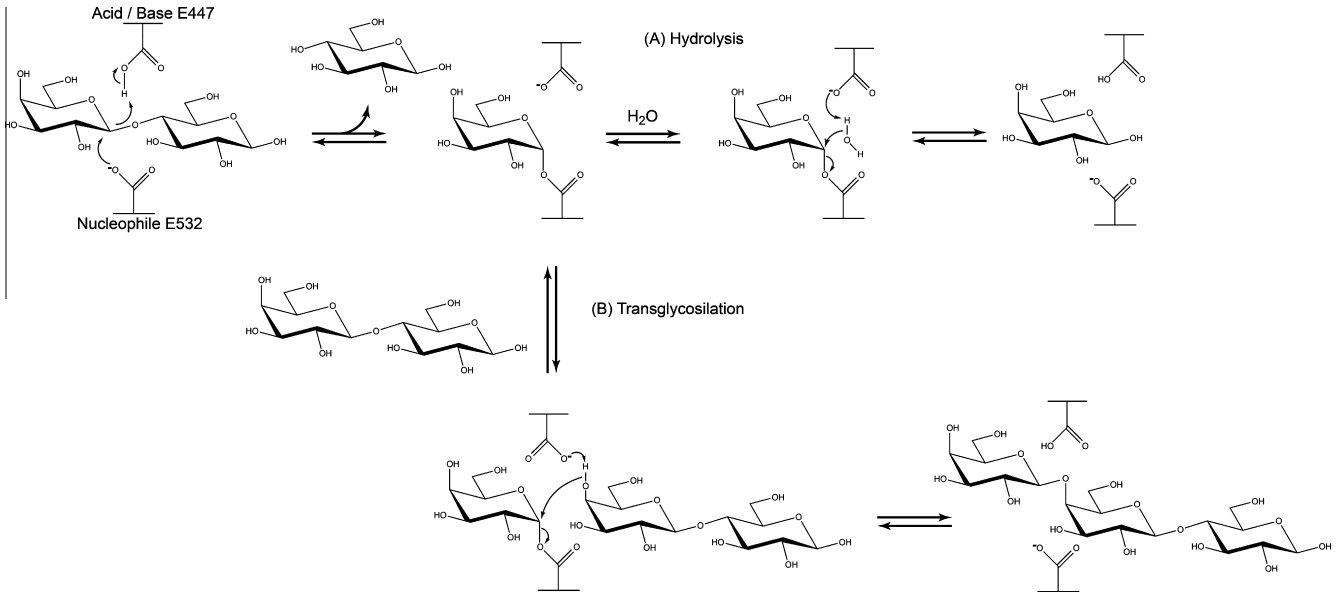

Fig. 1. Reaction mechanisms of the *B. circulans* β-galactosidase enzyme, also showing the catalytic glutamate residues involved, namely E447 as acid/base catalyst, and E532 as nucleophile. Residue H345 forms strong hydrogen bonds with the C3′ hydroxyl group of the galactosyl unit and stabilizes the transition state. The nitrogen of residue H379 is within H-bonding distance of the C3′ hydroxyl group of the galactosyl unit and thereby promotes catalysis by stabilizing the transition state (not visualized in the figure). (A) Hydrolysis reaction, using water as acceptor substrate. (B) Transglycosylation reaction, using lactose as acceptor substrate. Both reactions proceed according to a double-displacement mechanism and use lactose as donor substrates. The glycosidic linkage in lactose is cleaved, resulting in glucose release and covalent binding of galactose to the nucleophile in the active site. A subsequent reaction with water as acceptor substrate results in release of galactose as well (A: hydrolysis). Alternatively, lactose is used as acceptor substrate as well, resulting in release of oligosaccharide products (B: transglycosylation).

Table 1
Comparison of the β-galactosidase amino acid sequences of *Bacillus circulans* ATCC 31382 with other β-galactosidases.

Enzyme source	Enzyme name	No. a.a.[a]	Identity (%)	Similarity (%)	Uniprot code	PDB code[b]
Bacillus circulans ATCC 31382	BgaD-A	1737	100	100	E5RWQ2	
Bifidobacterium bifidum DSM 20215	BIF3	1752	28	43	Q9F4D5	
Bifidobacterium bifidum NCIMB 41171	BbgIII	1935	29	42	A4K5H9	
Bifidobacterium bifidum NCIMB 41171	BbgI	1291	14	24	E4V7B8	
Bifidobacterium bifidum NCIMB 41171	BbgIV	1052	13	23	Q0ZII7	
Kluyveromyces lactis CBS2359	BgaL	1025	13	22	P00723	3OB8
Escherichia coli K12	LacZ	1024	13	22	P00722	1DP0
Arthrobacter sp. C2-2	LacZ	1023	13	25	Q8KRF6	1YQ2

[a] Number of amino acids.
[b] PDB codes used for structural alignment with the 3D model of the rBgaD-cat (a.a. 50–650).

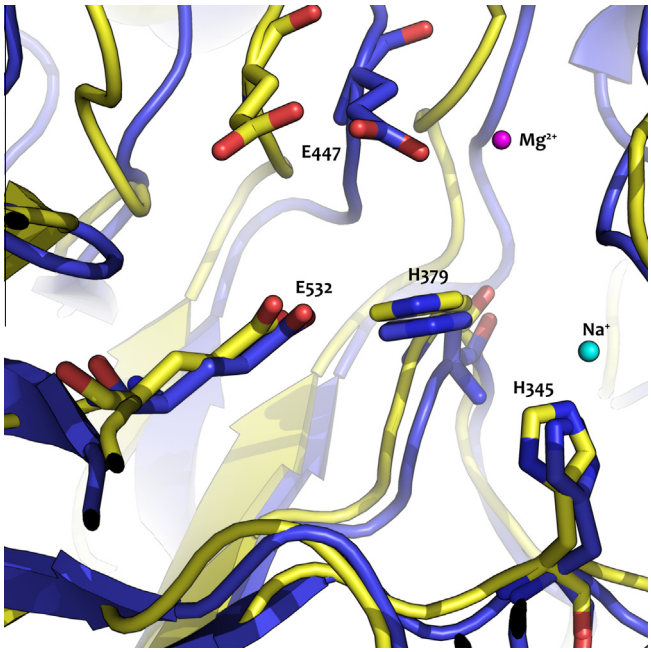

Fig. 2. Overlay of the protein model for *B. circulans* rBgaD-50–650 in yellow and *E.coli* β-galactosidase crystal structure (PDB: 1DP0) in blue. The two catalytic glutamates (E477, E532) and two conserved histidines (H345, H397) are shown for *B. circulans* rBgaD-50–650. The magnesium (magenta) and sodium (light blue) ions are required for *E.coli* β-galactosidase, but not for the β-galactosidase of *B. circulans*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

plasmids containing the desired mutation were transformed to *E. coli* BL21 (DE3) competent cells. For site-directed mutagenesis the following oligonucleotides were used in PCR reactions: 5′-CACG GC GTTTCGATGTTCCATGATTTAGGGG-3′ (H345F); 5′-CC ATCAGGGT TACCTTCAACCCGGCATCAC-3′ (H379F); 5′-ATCATG TG GTCGATCGG AAATAACATATATGATACGACCAATGCC-3′ (E447N) and 5′-CTGTAC GGCTCGCAGACGTCCTCGG-3′ (E532Q).

2.6. Recombinant protein expression and purification

E. coli expression of the β-galactosidase rBgaD-D and the four mutants were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Thermo) at an optical cell density at 600 nm of 0.6 for 4 h at 30 °C. Cells were harvested, washed with 20 mM Tris–HCl (pH 7.5) (Buffer A) and resuspended in B-PER lysis solution (Thermo). After 10 min incubation at room temperature, cell debris was removed by a centrifugation step and cell free extract with rBgaD-D was loaded on a HiTrap Q 1 ml column (Pharmacia) which was equilibrated with Buffer A. The flow through with rBgaD-D or one of the four mutants was collected, concentrated with an ultrafiltration device (Centricon Plus-20 with 50 kDa molecular cutoff, Millipore, Bedford, MA) and loaded on HiTrap Q 1 ml column (Pharmacia) which was equilibrated with Buffer B (20 mM N-Cyclohexyl-2-aminoethanesulfonic acid (CHES), pH 9.5) and unbound proteins were washed away with 5 column volumes of Buffer B. The elution was carried out with 20 ml of linear salt gradient to Buffer C (1 M NaCl in Buffer B). Fractions of 0.5 ml were collected and analyzed by 8% SDS–PAGE. Recombinant protein containing fractions were pooled and concentrated with an ultrafiltration device (Centricon Plus-20 with 50 kDa molecular cutoff, Millipore, Bedford, MA), loaded on a Superdex 200 column equilibrated with Buffer D (150 mM NaCl in Buffer A), and fractions of 0.5 ml were collected. Fractions with rBgaD-D or one of the four mutants were pooled, concentrated if necessary, and protein concentrations were determined as described.

2.7. Activity measurements

The specific activity of purified (mutant) β-galactosidase enzymes toward lactose was measured by following enzymatically released glucose with the glucose oxidase/peroxidase method (Megazyme). One enzyme activity unit is defined as the amount of enzyme in mg required for the hydrolysis of 1 μmol lactose per min at 40 °C and pH 6.0.

To determine kinetic parameters of (mutant) β-galactosidase, initial reaction rates for the hydrolysis of ortho-nitrophenyl-β-galactoside (o-NPG) with a molar extinction coefficient of 4500 M^{-1} cm^{-1} at 420 nm were determined spectrophotometrically (420 nm) at 40 °C and pH 6.0 over a concentration range of 1–38 mM. To prepare the substrate solutions, o-NPG was dissolved in 0.1 M phosphate buffer (pH 6.0) by heating and subsequently kept at 40 °C to prevent precipitation. The reaction was initiated by adding 10 μl of enzyme solution to 190 μl substrate solutions. The V_{max}, k_{cat} and K_M values, expressed as the mean ± SD of values obtained by triplicate measurements, were determined from Lineweaver–Burk (LB) plots in case of rBgaD-D and mutant E532Q.

2.8. Thermal shift assay

The melting temperature (T_m) (temperature at which 50% of the protein is unfolded) of rGalD-D and the four mutants derived were determined by a fluorescence-based thermal shift assay [21]. Solutions of 5 μl of 250 × SYPRO Orange (Molecular Probes), 2.5 μl of 1 M acetate buffer, pH 6.0, 12.5 μl of Milli-Q and 5 μl of 1 mg/ml protein were mixed, and heated from 25 °C to 95 °C in increments of 0.5 °C/min on a CFX96 real-time system-C1000 Thermal Cycler (Bio-Rad). All measurements were done in triplicate. SYPRO Orange dye interacts with a protein undergoing thermal unfolding, with its fluorescence increasing upon exposure to the protein's hydrophobic core. The T_M for rGalD-D and the four mutants were determined by

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BgaD_Bacci     341 GVSMHHDLG   372 VNAIRVTHNPAS   439 IIMWSIGNEIYD   528 LYGSETSSAT
BIF3_Bifbi     355 GVSMHHDQG   386 VNSIRTTHNPAA   471 VIMWSLGNEMME   561 IYGSETASAI
BbgIII_Bifbi   355 GVSMHHDQG   386 VNSIRTTHNPAA   471 VIMWSLGNEMME   561 IYGSETASAI
BbgI_Bifbi     466 GVNRHDSDP   497 VNAIRTSHYPNA   581 IIFWSMGNECAY   675 YVLCEFCHAM
BbgIV_Bifbi    374 GVNRHEFDC   405 INAVRTSHYPNQ   482 VLVWSLGNESYA   558 FVSCEYMHAM
BgaL_Klula     351 GVNRHDHHP   382 INAVRNSHYPNH   474 IIIWSLGNEACY   547 LILCEYGHAM
LacZ_Escco     354 GVNRHEHHP   385 FNAVRCSHYPNH   454 VIIWSLGNESGH   534 LILCEYAHAM
LacZ_Arthr     331 GVNRHETHP   362 VNAIRTSHYPPH   434 IVMWSLGNESGT   517 FILCEYVHAM
                   **  *           *  *  * *           ** ***           *   *
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Fig. 3. Multiple sequence alignment (created using T-Coffee and Jalview 2.8) of beta-galactosidase proteins, revealing four conserved blocks. The first two blocks contain the conserved histidine residues and the last two blocks contain the conserved and (putative catalytic) glutamate residues (boxed in grey). Asterisks indicate fully conserved residues. Residues subjected to mutagenesis are shown in bold [9,10,14,22]. BgaD_Bacci, *B. circulans* ATCC 31382 BgaD; BIF3_Bifbi, *Bifidobacterium bifidum* DSM 20215 BIF3; BbgIII_Bifbi, *Bifidobacterium bifidum* NCIMB 41171 BbgIII; BbgI_Bifbi, *Bifidobacterium bifidum* NCIMB 41171 BbgI; BbgIV_Bifbi, *Bifidobacterium bifidum* NCIMB 41171 BbgIV; BgaL_Klula, *Kluyveromyces lactis* CBS2359 BgaL; LacZ_Escco, *Escherichia coli* K12 LacZ; LacZ_Arthr, *Arthrobacter* sp. C2–2 LacZ.

Table 2
Kinetic parameters for the *Bacillus circulans* ATCC 31382 β-galactosidase rGalD-D and the E532Q site-directed mutant.[a] All parameters were determined at 40 °C and pH 6.0. One enzyme activity unit is defined as the amount of enzyme in mg required for the hydrolysis of 1 μmol lactose per min at 40 °C and pH 6.0. Hydrolysis of oNPG (oNP release at 420 nm) was used to determine the kinetic parameters V_{max} (μmol min^{-1} - mg^{-1} of protein), K_M (mM) and k_{cat} (s^{-1}). Subscripts high and low indicate parameters determined from the high and low substrate regions in biphasic Lineweaver–Burk plots, respectively. All measurements were done in triplo.

Enzyme	rGalD-D	E532Q
Lactose hydrolysis units min^{-1} mg^{-1}	158.5 ± 5.4	0.47 ± 0.02
oNPG hydrolysis kinetic parameters		
$V_{max,high}$	277.7 ± 2.7	1.0 ± 0.1
$K_{m,high}$	45.9 ± 2.7	43.6 ± 0.1
$k_{cat,high}$	424.1 ± 4.1	1.6 ± 0.1
$V_{max,low}$	35.8 ± 9.3	0.2 ± 0.01
$K_{m,low}$	1.5 ± 0.9	1.9 ± 0.3
$k_{cat,low}$	56.3 ± 11.9	0.2 ± 0.01

[a] H379F, H345F and E447N site-directed mutants were inactive.

calculating the first derivative from the melting curve using CFX manager 2.0 software (Bio-Rad).

3. Results and discussion

3.1. Alignment and structural modeling

The active site of β-galactosidase (LacZ) of *E. coli* is structurally and biochemically well characterized [8–15]. The amino acid residues E461 and E537 were identified as respectively the general acid/base catalyst and the nucleophile. Mutations in these residues, introducing several different residues at these positions, resulted in loss of activity [14,22]. Moreover, residues H357 and H540 were identified as being important for stabilization of the transition state. Mutations in these residues, introducing several different residues at these positions, also resulted in loss of activity [9,10]. Currently, no such information is available about the active site of *B. circulans* β-galactosidase rBgaD enzyme.

A protein sequence alignment was made between *B. circulans* β-galactosidase rBgaD and other β-galactosidases known to be producers of GOS (Table 1). The highest sequence identity was found with the BIF3 and BbgIII enzymes of *Bifidobacterium bifidum* DSM 20215 and *B. bifidum* NCIMB 41171, respectively (Table 1). However, also their active site residues have not been identified and characterized yet. The catalytic residues of β-galactosidases of *K. lactis*, *E. coli* and *Arthrobacter* sp. C2-2 were previously identified but their sequence identity (Table 1) was too low for identification of the catalytic residues of *B. circulans* β-galactosidase. To improve this protein sequence alignment, a 3D protein model was predicted for the catalytic domain of *B. circulans* rBgaD-50–650 (a.a.) and structurally aligned with crystal structures of *K. lactis* BgaL, *E. coli* LacZ and *Arthrobacter* sp. C2-2 LacZ (Fig. 2). Manual adjustments based on the structural alignment were made and enabled the identification of the rBgaD-D conserved residues E447 and E532 as the putative general acid/base catalyst and the nucleophile, respectively. The two histidines in *E. coli* LacZ identified as being important for stabilization of the transition state were also conserved in *B. circulans* with H345 and H379 as their equivalents. Moreover, two conserved glutamates, the putative general acid/base catalyst and the nucleophile for *B. bifidum* DSM 20215 BIF3 (E479 and E567), *B. bifidum* NCIMB 41171 BbgI, BbgIII and BbgIV (E589 and E681, E479 and E567, E490 and E564, respectively) which have not been reported before, were identified as well (Fig. 3).

3.2. Characterization of site-directed mutants

B. circulans β-galactosidase residues H345, H379, E447 and E532 were changed by site-directed mutagenesis. The activity of the purified enzymes with lactose was determined by measuring glucose release. Three mutants (H345F, H379F and E447N) were inactive with the assay used, whereas the activity of mutant E532Q was reduced at least 340-fold compared to the wild type enzyme (Table 2). This is the first report on mutagenesis of these residues of the *B. circulans* ATCC 31382 β-galactosidase, showing that when they are changed, enzyme activity is lost (virtually) completely.

Kinetic properties could only be determined for wild type and site-directed mutant E532Q (Table 2). Both wild type and E532Q mutant showed biphasic Lineweaver–Burk (LB) plots for which kinetic parameters could be determined separately. This resulted in two sets of kinetic parameters (V_{max}, K_M and k_{cat}): one for the lower and one for the higher substrate concentration regions, similar to what was shown previously for rGalD-D [3]. Such biphasic LB plots have been reported for hydrolytic enzymes that show transglycosylation activity at higher substrate concentrations [2,6]. The $k_{cat,high}$ for the E532Q mutant had decreased 270-fold, with an almost unchanged $K_{m,high}$. A decreased $k_{cat,low}$ value (250-fold) also was determined for the E532Q mutant, whereas its $K_{m,low}$ increased about 30% (from 1.5 to 1.9 mM). The assay sensitivity could not be increased enough to determine kinetic parameters for the three other mutants.

On basis of this data we conclude that E447 and E532 of *B. circulans* β-galactosidase act as general acid/base catalyst and nucleophile, respectively. The data also shows that H345 and H379 are essential for activity of the *B. circulans* β-galactosidase. This strongly suggests that these two residues are important for transition state stabilization as was shown for the similar residues in the *E. coli* β-galactosidase [9,10].

3.3. Melting temperatures (T_m) determined with the thermal shift assay

Mutations in the catalytic residues and two histidines of the *E. coli* β-galactosidase caused minor, if any, structural changes in

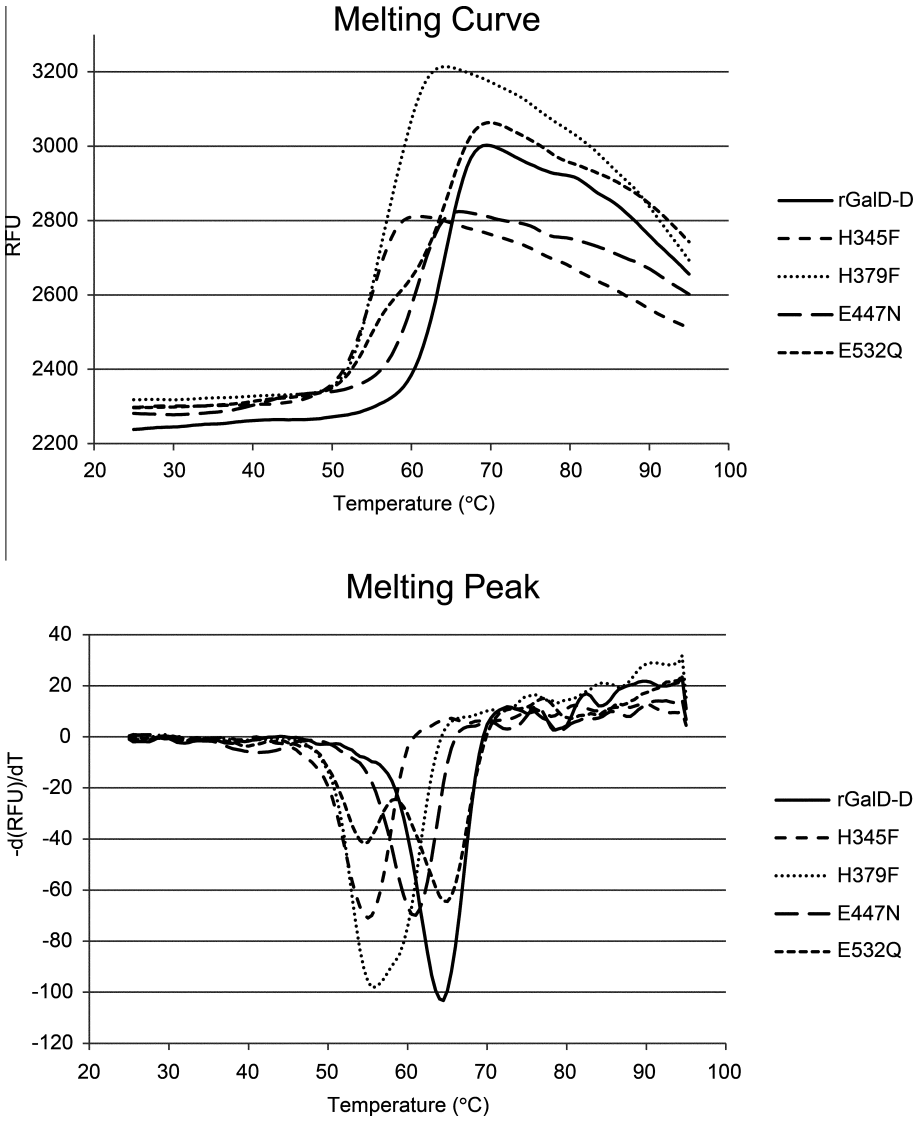

Fig. 4. (Top) Melting curves showing protein unfolding by increasing temperatures, accompanied by increase in SYPRO Orange fluorescence. The melting temperature (T_m) values were determined by calculating the first derivative from the melting curve with CFX manager 2.0 software (Bio-Rad). (Bottom) The *X*-axis value (*T*) that corresponds with the lowest *Y*-axis value (−d(RFU)/dT) in the resulting curve resembles T_m. In the case of E532Q two melting temperatures were found.

the protein [9,10,15,22]. To determine whether mutations in the catalytic residues and two histidines of the *B. circulans* ATCC 31382 β-galactosidase had caused structural changes; we recorded protein melting curves for rGalD-D and mutant enzymes derived and determined the melting temperatures (T_m). All 5 enzymes showed typical melting curve profiles (Fig. 4). The T_m values obtained are shown in Table 3. Introduction of mutations H345F and H379F caused a decrease of 9 °C in T_m values whereas mutation E447N lowered the T_m by 3.5 °C. Interestingly, the E532Q mutant enzyme showed 2 distinct melting temperatures, the first one is 10 °C lower than the T_m value for rGalD-D, the second one being equal to the T_m value for rGalD-D. This observation was only made for mutant E532Q and may be explained by successive melting of independent protein domains caused by the mutation E532Q. From these results we conclude that the mutant proteins were properly folded, however, introduction of point mutants in and close to the active site somewhat decreased the thermal stability of the proteins.

Table 3
Melting temperatures (T_m) determined with the thermal shift assay. The melting temperature (T_m) represents the temperature at which 50% of the protein is unfolded [21].

Enzyme	T_m (°C)	T_m (°C)
rGalD-D	64.3 ± 0.3	–
rGalD-D H379F	55.7 ± 0.3	–
rGalD-D H345F	55.2 ± 0.3	–
rGalD-D E447N	60.8 ± 0.3	–
rGalD-D E532Q	54.7 ± 0.3	64.8 ± 0.3

In conclusion, active site residues in the *B. circulans* β-galactosidase essential for catalysis and activity have been identified. Such information is of prime importance for our future attempts to change enzyme properties. Our current work provides a firm basis for enzyme engineering to further investigate the reaction mechanism.

Author contributions

JBB designed the experiments, conducted the experiments and wrote the paper, BJHK wrote the paper, LD designed the experiments and wrote the paper.

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