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Role of N166 residue in β–glucosidase catalysis and glucose tolerance

Abstract

Product inhibition of β-glucosidase is one of the limiting factors in complete saccharification of lignocellulosic biomass to fermentable sugars. Relieving the detrimental effects of glucose on β-glucosidase will, therefore, be of great economic interest for the cost-effective deconstruction of recalcitrant biomass. The indispensable role of β-glucosidase in saccharification of cellulose led to the identification of GH1 family of CAZymes which have increased glucose tolerance and are stimulated in the presence of glucose, however, the exact mechanism is still elusive. The present study aims at unravelling interaction of glucose with active site residues in β-glucosidase (Gluc1C) using the combinatorial approach. Computational analysis revealed that amongst the catalytic centre residues, N166 interacts exclusively with glucose and no interaction was observed with substrate analogue thiocellobiose. In order to further understand the putative role of this residue in catalysis and product inhibition, we generated six single site-directed mutants. Biochemical characterization of the mutants indicated that of the six mutants only N166D mutant was active against para-nitrophenol-β-D-glucopyranoside (pNPG) as substrate. The N166D mutant was highly stimulated at 100mM glucose and was having a marginal increase in affinity for substrate pNPG, whereas replacement of N166 with amino acids with bulkier side chains resulted in a complete loss in activity. Thus, signifying the role of N166 in positioning substrate accurately between +1 and -1 subsite for hydrolysis as well as in substrate/ inhibitor binding and stimulation. The present study, highlight that apart from the regions around the active site entrance, the residue adjacent to an active site plays an important role in modulating the binding preference for cellobiose and glucose. The aforesaid study highlights the mechanistic details for glucose binding preferences in the enzyme active site. The study will also aid in rational engineering and selection of β-glucosidase for diverse biotechnological applications.

Keywords: β-glucosidase, glucose tolerance, stimulation, mutagenesis

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Introduction

β-glucosidases are a class of enzyme that catalyze the hydrolysis of β-1,4- glycosidic linkages thereby releasing non-reducing terminal glucosyl residues from glycosides and oligosaccharides. Owing to their crucial role in saccharification of cellulosic biomass, β-glucosidases have recently gained huge attention. In a typical biomass saccharification reaction, β-glucosidase works synergistically with other cellulases (endocellulases and cellobiohydrolases) to convert insoluble cellulose into fermentable sugars.[1,2] In order to make the entire process economically viable, it is advisable to operate the cellulose hydrolysis at higher dry matter consistency. This results in release of cello-oligosaccharides and cellobiose (hydrolysis products) at high concentration during enzymatic reaction. The accumulation of higher cello-oligosaccharides and cellobiose results in product inhibition of processive cellobiohydrolases and endoglucanases stalling the entire saccharification reaction. β-glucosidase are known to overcome this stalling by cleaving cellobiose to glucose thereby relieving the inhibitory effect on cellobiohydrolases and endoglucanases. However, β-glucosidases is highly sensitive to the inhibitory effects exhibited by glucose which results in thereafter accumulation of cello-oligosaccharides and cellobiose leading to product inhibition issues.[3,4] Thus, for commercial application, ideal β-glucosidase candidate for supporting cellulases in cellulosic conversion should have high catalytic efficiency along with strong glucose tolerance.[5] The presently available β-glucosidase lacks thermal stability, possesses narrow pH range and product inhibition by glucose.[6] The discovery or development of glucose tolerant β-glucosidase is therefore, of considerable interest because it can lead to cellulases and β-glucosidase usage at an economic dosage to attain acceptable yields during cellulose hydrolysis.[1,7]

Nature has presented the diverse set of β-glucosidases, some highly sensitive, some simulated or highly tolerant to glucose concentration. Despite the presence of β-glucosidases with varied glucose tolerance, the current understanding of glucose inhibition and its structural basis is still elusive. Various mechanistic models for glucose inhibition and tolerance have been proposed implicating the glucose tolerance of β-glucosidase with glucose simulation and/ or transglycosylation.[8,9] However, the reported mechanism does not follow 'one size fits all' approach and may vary amongst β-glucosidases.

Various approaches have been used to understand the mechanistic details of β-glucosidases catalysis and for enhancing the catalytic properties of enzyme.[10,11] Directed as well as rational protein engineering strategies have been well documented for increasing the thermal stability and altering pH optima of β-glucosidases.[12–14] while others have reported structure-function relationship of β-glucosidases with respect to substrate specificity and transglycosylation.[15,16] Few studies have elucidated the structure-function relationship with respect to glucose tolerance at the aglycone site are recently been reported. In one such study, Liu et al.,[5] studied the aglycone region of the active site of *Paenibacillus* β-glucosidase and indicated

the role of H184 in glucose tolerance. Corresponding mutation of this residue to hydrophobic aromatic group resulted in decreased activity with 20% increased glucose tolerance with no evidence for mechanism for enhanced glucose tolerance. In another recent study, Yang et al reported that specific sites at the entrance and middle of the substrate channel of β-glucosidase regulate the effects of glucose and the relative binding affinity/preference of these sites towards glucose modulates the glucose dependence.[17] Both the studies highlight the significance of aglycone region for modifying glucose binding preference. However, glucose being a competitive inhibitor of β- glucosidase, studying its interaction at the active site/glycone is crucial in order to understand its interaction pattern at the active site. Till date, all the studies indicating a structural basis for glucose tolerance have been carried out by engineering the aglycone subsite of β-glucosidases and fails to explain its interaction at the active site/ glycone site. In the present study, using structural modeling and site-directed mutagenesis, we studied the role of N166 a conserved glycone pocket residue in modulating glucose tolerance of enzyme. The study provides a starting point for the rational engineering of β-glucosidases for improvising their glucose tolerance for industrial application.

Material and methods

Materials, strains and media

Escherichia coli DH5α strain [F-Φ80lacZΔM15Δ(lacZYA-argF) U169 recA1endA1hsdR17 (rK–,mK +)phoAsupE44λ–thi-1 gyrA96 relA] was used for genetic manipulations. Glu1C β-glucosidase gene was used for mutagenesis was the product of our previous study.[18] *E. coli* was cultured in Luria-Bertani (LB) medium (10g/l tryptone, 5g/l yeast extract, and 10g/l NaCl) supplemented with 100μg/ml ampicillin. DNA purification, extraction kits and Ni-NT Agarose for His-tag protein purification were purchased from Qiagen. All other chemicals used in the study were of analytical grade.

Bioinformatics analysis

Currently no crystal structure is available for Glu1C β-glucosidase from *Paenibacillus polymyxa*. However, crystal structure of BglB β-glucosidase also isolated from *Paenibacillus polymyxa* is available in Protein Database with PDB code: 2O9T & 2O9R containing glucose and thiocellobiose respectively at the active site.[19] These structures were therefore used as a template for the in silico mutagenesis study. The sequence alignment of Glu1C with BglB sequence from *Paenibacillus polymyxa* was performed using EMBL-MUSCLE, while interaction profiles of ligand with the enzyme at the active site were calculated using PDB-Ligand Protein Contact[20] and mBLOSSUM analysis.[21]

In silico mutation studies

Molecular dynamics simulations were performed by the Standard Dynamics Cascade protocol of Discovery Studio 2.5(Accelrys, CA, USA). The structures of the wild-type and the mutants modelled in this study were used as starting models for MD simulations. The CHARMm force field was used for modelling. The heating, equilibration, and production steps of the MD simulation were performed with default parameters except the energy minimization step. The first and second energy minimizations were carried out by steepest descent and conjugate gradient algorithms, respectively and the maximum steps were set to 5000. MD simulations were carried out for 1.0ns and the atomic trajectories of β-glucosidases were saved every 10 mins for structural analysis. A total of 10 trajectories were used for the analysis. Structure with the lowest energy was selected and compared with the wild type β-glucosidases. All MD simulations were performed at 300K.

Site directed mutagenesis

Quick Change ™ site-directed mutagenesis protocol[22] was applied to construct the mutants. All primers used in this study are listed in Table 1. The mutant plasmids were transformed in *E.coli DH5* α cells and selected on LB agar plate supplemented with 100μg/ml of ampicillin. The positive clones were picked and cultured in 5mL LB media supplemented with 100μg/ml ampicillin. The mutant plasmids were extracted and confirmed by gene sequencing.

Table 1 List of Primer sequences used in PCR Mutagenesis

Primer Name	Primer sequence (5'-3')
Glu1C N166D -F	CTGGTGGAATACGATCGATGAGCCTTATTGCGCC
Glu1C N166D -R	GGCGCAATAAGGCTCATCGATCGTATTCCACCAG
Glu1C N166M -F	CTGGTGGAATACGATCATGGAGCCTTATTGCGCC
Glu1C N166M -R	GGCGCAATAAGGCTCCATGATCGTATTCCACCAG
Glu1C N166Q -F	CTGGTGGAATACGATCCAGGAGCCTTATTGCGCC
Glu1C N166Q-R	GGCGCAATAAGGCTCCTGGATCGTATTCCACCAG
Glu1C N166F -F	CTGGTGGAATACGATCTTTGAGCCTTATTGCGCC
Glu1C N166F -R	GGCGCAATAAGGCTCAAAGATCGTATTCCACCAG
Glu1C N166R-F	CTGGTGGAATACGATCCGTGAGCCTTATTGCGCC
Glu1C N166R -R	GGCGCAATAAGGCTCACGGATCGTATTCCACCAG
Glu1C Y298H -F	GGGCATTAACTATCATACCCGCAGCATCATTCG
Glu1C Y298H -R	CGAATGATGCTGCGGGTATGATAGTTAATGCCC

Expression and purification of enzymes

The wild-type and mutant clones of β-glucosidases were grown in 500ml LB medium supplemented with 100μg/mL ampicillin at 37ºC until absorbance at 600nm reached to 0.5-0.6. Cells were induced by adding 1mM IPTG and cultured at 20ºC for 16 hours to express the β-glucosidases. The cells were harvested by centrifugation. The cell pellets were suspended in Lysis buffer (50 mM Na_2HPO_4, 300mM NaCl and 10mM Imidazole pH 8.0) and the cells were lysed via sonication. The supernatants were recovered by centrifugation (8000 RPM for 45minutes) and recombinant proteins were purified on Ni-nitriloacetic acid (NTA) resin (Qiagen) as per the manufacturer's guidelines. The purified fractions were pooled, dialyzed against the

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assay buffer, and used for further characterization. Enzyme purity was estimated by 12% SDS-PAGE and enzyme concentration was determined by Bradford assay.

Enzyme assays

β-glucosidase activity was determined by incubating the enzyme with 5mM para- nitrophenyl-D-glucopyranoside (pNPG) in 50mM Citrate buffer (pH 6.0) in 0.55ml at 50°C for 15 minutes. The reaction was stopped by adding 1ml of 1% Sodium carbonate, and the absorbance was measured at 400nm. One unit of β-glucosidase activity was defined as the amount of enzyme that produced 1µmol of para- nitro phenol per minute.[23]

Measurement of pH, temperature optima and thermostability of the enzyme

The activities of purified recombinant enzymes were measured under different pH conditions to determine the optimal pH. Four buffers of various pH ranges- 50mM citrate phosphate buffer (pH 3.0 to 6.0), 50mM sodium phosphate buffer (pH 6.0 to 7.0), 50mM Tris-HCl buffer (pH 7.0 to 9.0) and 50mM carbonate buffer (pH 10.0)- were used in the assay and the enzyme activity was determined as described above. The optimal temperature for enzyme activity was determined by incubating reaction mixtures over a temperature range of 20°C to 70°C and determining their activity. The thermal stability of β-glucosidases was determined by incubating the enzymes at various temperatures for 30 minutes, cooling them down to ambient temperature, and then initiating the reactions by addition of substrate. The formation of para- nitrophenol was determined as described before in Enzyme assay section.

Circular Dichroism Spectrometry

Far UV-CD spectrum data was collected on MOS-500 circular dichroism spectrometer (Bio-Logic Science Instrument, Claix, France) in 1mm and 2mm path length cuvette with protein concentration of 0.54mg/ml for native Glu1C and 0.03mg/ml for N166D mutant β-glucosidase in 50mM Na phosphate buffer pH 6.0 respectively. Obtained raw ellipticity was converted into mean residue ellipticity for plotting.

Measurement of kinetic parameters

For determination of Km and *V*max, five different substrate concentrations were used in the range of 0.15 to 10mM pNPG for β-glucosidase. The Km and *V*max were determined directly from the hyperbolic curve fitting of the Michaelis- Menten equation.

Glucose tolerance

The effect of glucose on the β-glucosidase activity was evaluated by testing the activity of β-glucosidases towards pNPG in the presence of glucose (0 to 2000 mM). The Ki was determined by plotting 1/V against 1/[S] for the enzymes in the presence of different concentrations of glucose and fitting the data with Eq. 1 in which [I] represents the concentration of glucose.

$$\frac{1}{v} = \frac{Km}{Vmax}\left(1+\frac{[I]}{Ki}\right)\frac{1}{[S]} + \frac{1}{Vmax} \qquad \text{..Eq.1}$$

Results

Selection of target residue and molecular modelling

Multiple sequence alignment studies revealed that previously cloned and characterized Glu1C β-glucosidase from *Paenibacillus polymyxa* showed 99% sequence similarity with BglB β-glucosidases. Two BglB structures, 2O9T and 2O9R containing glucose and thiocellobiose in the active site pocket were selected for Ligand Protein contact analysis and mBLOSSUM analysis. Table 2 highlights participation of total of 13 amino acids to make active site pocket, of which Glu 167 and Glu 356 represents catalytic acid/base residue and catalytic nucleophile respectively resulting in cleavage of β-1,4 glycosidic linkage in retaining configuration. In addition to catalytic residues, the active site is made up of hydrophobic aromatic residues such as Phe, Tyr, Trp which promotes stacking of carbohydrate ring structure at the active site pocket.[19] Interestingly, the ligand protein contact analysis showed up one of the residues in active site pocket, N166 to interact with glucose via H-bonding while no interaction was observed with thiocellobiose (Figure 1) (Table 2). This prompted us to study the role of N166 in detail to unravel the probable function of this residue at the active site pocket. We performed saturation mutagenesis at N166 in silico and studied the change in interaction and internal energy.

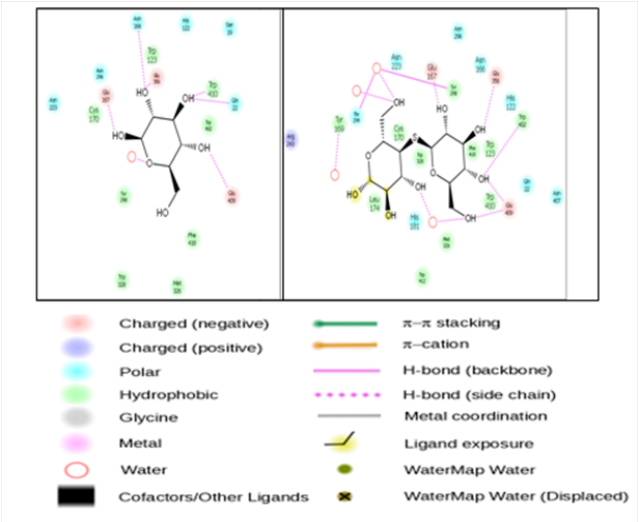

Figure 1 Interaction profile diagram of (a) glucose and (b) thiocellobiose with active site residues of β-glucosidases from *Paenibacillus polymyxa*.

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Table 2 mBlossum and Ligand Protein Contact (LPC) analysis of crystal structure of BglB β-glucosdiase from Paenibacillus polymyxa 2O9T (with glucose) and 2O9R (with thiocellobiose)

Aminoacid	Location	Statistics	Mutability Score	Glucose	Thiocellobiose
Q22	Pocket	50xQ	1	1	1
H122	Pocket	50xH	1	2	1
W123	Pocket Tunnel	50xW	1	3	4
N166	Pocket	50xN	1	1	no interaction
E167	Catalytic site	50xE	1	6	6
N296	Catalytic Pocket	Nx50	1	6	8
M 326	Pocket Tunnel	40xM,6xI,2xF,2xR	2	2	2
W 328	Pocket Tunnel	50xW	1	5	17
E 356	Cataytic Pocket Tunnel	50xE	1	5	3
W 402	Pocket	50xW	1	5	7
E 409	Pocket	50xE	1	4	7
W 410	Pocket Tunnel	50xW	1	4	6
F 418	Pocket	50x F	1	4	4
C 170 (2O9T)	Pocket Tunnel	40xC,10xV	2	1	0
H 181 (2O9R)	Pocket Tunnel	44xH,4xF,1xM,1xQ	1	0	7

The choice of stable mutants was made taking the ligand in consideration, i.e. in case of glucose (inhibitor), the mutant giving highest interaction energy (unstable) with glucose, while for thiocellobiose (Substrate analog), the mutant giving lowest interaction energy (Stable) were selected. Based on these criteria, N166D, N166Q and Y298H were selected (Figures 2 (A& B)). However, it was found that in case of wild type enzymes when both glucose and thiocellobiose is in the active site pocket, the interaction energy of the system was positive and hence the system was highly unstable. The same phenomenon was observed for mutant N166M indicated by an orange circle in Figure 2 (C). Further internal energy of the system was calculated, with glucose, thiocellobiose, and glucose with thiocellobiose together at the active site pocket. The wild type enzyme was having an internal energy of -145.96KJ/mol, -249.98KJ/mol,-402.336KJ/mol with glucose, thiocellobiose, and glucose with thiocellobiose at the active site pocket. Keeping the wild type as the reference, the mutants which were given a value similar to extremely lower energy (Stable) were chosen. Thus, based on internal energy, N166R, N166D, N166Q, and N166F were selected. Six mutants namely Y298H, N166R, N166D, N166Q, N166M, and N166F were selected for in vitro Site-directed mutagenesis. The mutated proteins were isolated from 500ml culture broth and purified using Ni-NTA affinity chromatography. The mutant proteins were then characterized with respect to activity, thermal stability, and glucose tolerance.

Biochemical and biophysical characterization of N166 mutants

The selected ß-glucosidase mutants were expressed and purified using Ni-NTA affinity chromatography. Three mutants N166D, N166Q, and N166R were successfully purified (Figure 3). Table 3 indicates the specific activities of the wild type and mutant enzymes of which the only N166D showed 20% activity of wild type Glu1C. The effect of pH on the activity of wild type Glu1C and N166D mutant enzyme was tested in the pH range of 2.0- 8.0. The mutant protein showed a similar activity profile as the wild type with the enzyme showing optimal activity at pH 6.0 (Figure 4). The optimal temperature for β-glucosidases was determined by measuring the initial activity for 15minutes at different temperatures from 20ºC to 70ºC. The optimum temperature for N166D was about 50ºC and the activity profile was similar to wild type Glu1C enzyme (Figure 4 (B)). The thermostability of N166D was further investigated by measuring the resistance to heat activation at 55ºC for one hour. N166D displayed four-fold increased stability at 55ºC compared to wild type Glu1C with an increased half-life (t1/2) at 55ºC from 5mins to 20 mins for the N166D mutant enzyme (Figure 4 (C)). The glucose tolerance ability of the wild type and N166D mutant was examined using pNPG as substrate. The wildtype Glu1C β-glucosidase was 50% active at 1M glucose while the mutant N166D displayed 75% activity at 1M glucose for a period of 3 hours. However, both the wild type and mutant enzymes were completely inhibited when incubated with 1M glucose for 5 hours (Figure 4). The increase in glucose tolerance was further indicated by an increase in Ki from 4.5 to 14.5mM for glucose calculated using Lineweaver Burk plot (Figure 4). The results implied that N166 residue might play role in thermal stability as well as glucose tolerance in β-glucosidases catalysis. In order to support the data for the change in structure, we performed CD analysis of the mutant and wild type enzyme. A considerable shift at 280nm suggested an increase in alpha helical content in mutant N166D as oppose to native Glu1C (Figure 5). This shift suggests increased in α- helical content of the protein secondary structure, thereby resulting in increased thermostability.

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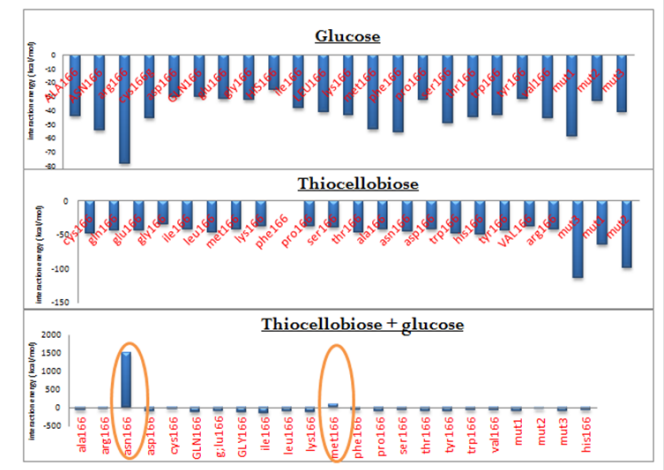
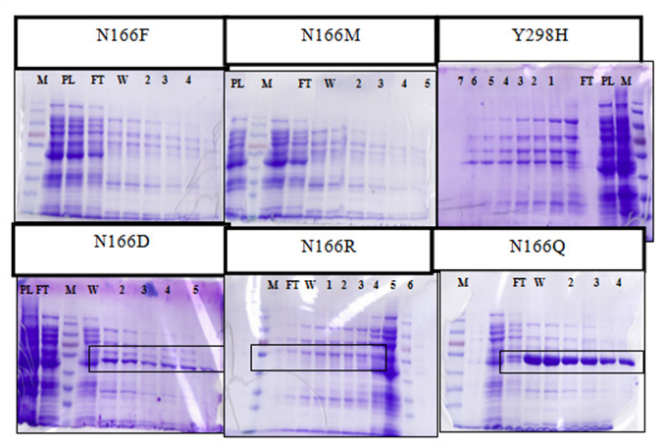


Figure 2 Interaction energy calculations of all mutant β-glucosdiases with glucose (a), thiocellobiose (b) and thiocellobiose and glucose (c) at the active site.



Abbreviations: lane PL, preload; lane FT, flowthrough; lane W, wash; lane 1-7, elution fractions 1-7; lane M, molecular mass marker.

Figure 3 SDS PAGE gel of Purification profile of recombinant Glu1C mutant enzymes after affinity purification.

Table 3 Specific activity and Kinetic properties of mutant and wild type Glu1C β-glucosidase

Mutant	pNPG Activity (U/mg)	Km (mM)	Vmax (mM min-1)	Ki (mM)
N166D	3.018±0.5	3.18±0.6	0.289±0.5	14.43±0.5
Native	15.00± 1.5	4.17±0.7	5.85±0.5	4.49±0.5

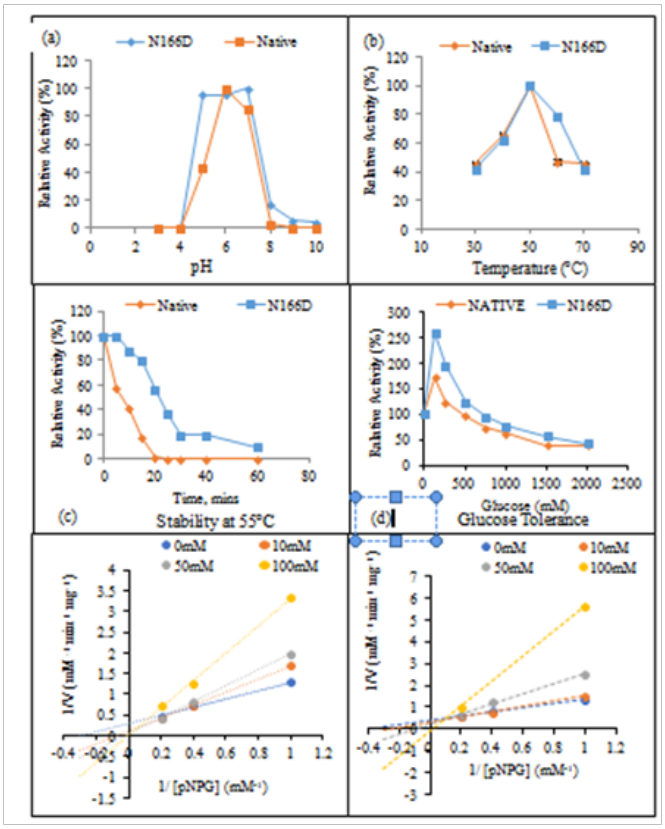


Figure 4 Comparative Profiles for pH optima (a), Temperature optima (b), Thermal Stability at 55°C (c) and Glucose tolerance (d) between Native Glu1C enzyme and N166D mutant purified enzyme. Inhibition Kinetics of Native Glu1C (e) and N166D (f) with Glucose.

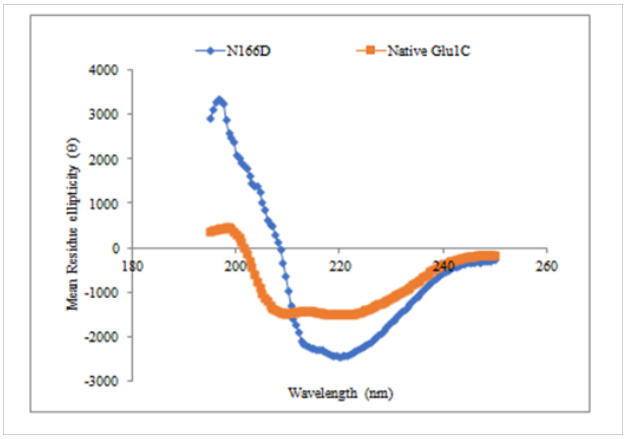


Figure 5 CD spectra for Native Glu1C and mutant N166D recorded in far UV regions showing considerable increase in α- helical content.

Discussion

The cost-effective production of cellulosic ethanol demands operation at higher dry matter consistency in order to achieve high glucose yields. This necessitates usage of robust enzymes able to tolerate higher concentration of cello-oligosaccharides intermediates

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and glucose. The currently employed cellulolytic systems derived from *T. reesei* are deficient in key enzyme- β-glucosidase and are highly prone to inhibitions by hydrolysis product cellobiose and glucose.[9,10] The inhibition issue becomes more prevalent at higher biomass loading due to viscosity and mass transfer limitation by accumulating intermediates which limits the overall conversion efficiency and resulting glucose yields. Glucose exhibits its inhibitory effects indirectly on other enzymes while in case of β-glucosidase, glucose exhibits its inhibitory effect directly resulting in slow rate of catalysis. This necessitates understanding the mechanistic features of glucose inhibition particularly with respect to β-glucosidase. Glucose is known inhibit β-glucosidase via competitive inhibition mechanism however, non-competitive and mixed inhibition is also reported. The diversity in mechanism of inhibition is due to activation of β-glucosidase at low glucose concentration while inhibition at higher concentratin.[9–11,17]

Numerous theories explaining glucose inhibition have put forward generalizing mainly two approaches, firstly glucose can inhibit the enzymatic activity of β-glucosidase by competing with cellobiose for binding directly to the active site. Secondly, glucose could affect the active site indirectly by binding to the aglycone site thereby perturbing the water matrix and steric geometry in the substrate channel.[17] Notably, all the mutagenesis study carry out till date focuses on the second approach wherein the aglycone pocket of the β-glucosidase has been subjected to mutagenesis.[15,16,21,24] In the present study, we elucidated the importance of N166 active site residue interacting with glucose directly at the active site and its role in modulating the activity and glucose tolerance. Sequence- structure analysis and molecular modelling suggested that the active site cleft of Glu1C β-glucosidase contains the primary motif of T-F-N-E-P and I-T-E-N-G which is highly conserved amongst family I β-glucosidase.[15] N166 residue selected for mutagenesis belongs to the motif T-F-N-E-P and hence conserved amongst Family I β-glucosidases. Ligand protein contact analysis showed that apart from one residue N166, all the amino acid residues framing the active site pocket were making contact with both glucose as well as thiocellobiose. This conserved N166 residue was making a single contact with Glucose and no contact with Thiocellobiose. This prompted us to do saturation mutagenesis at N166 residue for elucidating its role in β-glucosidase catalysis.

The results indicated that a single point mutation at the active site residue not only affected the activity of the mutant enzyme but also purification of the enzymes. Biochemical characterization of the three purified mutants N166D, N166Q, and N166R demonstrated that replacement of the same length but opposite charge (Asn-Asp) at 166 position resulted in 20% activity of wild type, while a longer or shorter side chain resulted in complete loss of activity. One possibility for this could be that presence of bulkier side chain like Arg, Phe, Met in the vicinity of catalytic residues might perturb the environment of these residue resulting in less favorable catalytic geometry.[25] These bulkier side chains restrict the glycosyl group of the substrate in +1 subsite rather than in between +1 and -1 subsites, due to which the enzyme is not able to catalyze the hydrolysis of a β-1,4 glyosidic bond (Figure 6). The position 166 (adjacent to the active site) thus, was highly sensitive to the charges and length of the amino acid side chain.

Of the Glu1C variants examined, N166D showed increased thermostability at 55ºC. Many studies have implicated this type of interaction in protein stabilization wherein the elimination of labile residues like Met and Asn, which can undergo oxidation or deamination at high temperature making the enzyme irreversible to thermal denaturation.[26] The N166D mutation also resulted in an increase in affinity for the pNPG substrate which is in good agreement with the in silico modelling experiment. However, the mutation resulted in 20 fold decrease in the Vmax. This decrease in the rate might be due to the repulsive force of the Asp 166 residue for the substrate. Glucose tolerance analysis showed that the N166D mutant was highly stimulated at 100mM glucose concentration and showed 10% increase tolerance to glucose as opposed to wild type Glu1C. The increased affinity for the substrate and stimulatory effect of glucose observed in case of the N166D mutant is well explained by Yang et al, wherein they proposed the preferences of glucose to occupy a site distinct from the active site enhancing substrate cleavage activity via transglycosylation or other mechanisms.[17] Changes in Far UV CD spectra at 280 nm suggest a change in secondary structure specifically in the alpha helical content of the protein. The thermostability and changes in affinity for pNPG and glucose might be due to this change in secondary structure of native protein.[27]

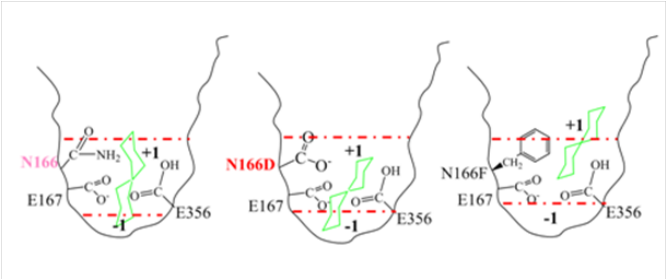

Figure 6 Diagrammatic representation of change in active site due to mutation at N166 residue. The cellobiose is represented in green between +1 and -1 subsite for hydrolysis while when mutated N166F restricted at +1 subsite.

In summary, a single point mutation at the active site resulted in increased thermal stability as well as a marginal increase in glucose tolerance with compromised activity. The relative loss in activity is due to narrowing of active site pocket, which hindered the rotation of glycosidic bond in favorable catalytic geometry. Thus, at the active site, not only charges but also the length of mutated residues plays a key role in determining the efficacy of the enzyme.

Conclusion

Enzymatic saccharification of cellulosic biomass at high solid loading is necessary for generating concentrated glucose solution which will ease the downstream conversion of sugar to fuels and chemicals. Under this condition, swift and efficient removal of glucose is crucial thereby preventing the enzyme from adverse effects of product inhibition. Our work demonstrates that alleviating product inhibition in β-glucosidase requires a delicate balance between maintaining activity for glucose production and allowing it to escape efficiently.

The results reveal a trade-off between catalytic activity and product tolerance in an attempt to alleviate glucose inhibition in β-glucosidase. The study also indicated that apart from the aglycone site, active site residue also participates in stimulatory effect and tolerance to glucose in β-glucosidases catalysis. Therefore, development of glucose tolerant β-glucosidase not only requires modulating the entrance and pockets around the active site but residues surrounding the active site that also plays a significant role in glucose tolerance and stimulation.

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Acknowledgments

None.

Conflicts of interest

The author declares there are no conflicts of interest.

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