

Influence of *N*-glycans on Expression of Cell Wall Remodeling Related Genes in *Paracoccidioides brasiliensis* Yeast Cells

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Abstract: Paracoccidioidomycosis is the most prevalent systemic mycosis in Latin America. It is caused by the temperature-dependent dimorphic fungus *Paracoccidioides brasiliensis*. The *P. brasiliensis* cell wall is a dynamic outer structure, composed of a network of glycoproteins and polysaccharides, such as chitin, glucan and *N*-glycosylated proteins. These glycoproteins can interact with the host to affect infection rates, and are known to perform other functions. We inhibited *N*-linked glycosylation using tunicamycin (TM), and then evaluated the expression of *P. brasiliensis* genes related to cell wall remodeling. Our results suggest that cell wall synthesis related genes, such as β -1,3-glucanoyltransferase (*PbGEL3*), 1,3- β -D-glucan synthase (*PbFKS1*), and α -1,4-amylase (*PbAMY*), as well as cell wall degrading related genes, such as *N*-acetyl- β -D-glucosaminidase (*PbNAG1*), α -1,3-glucanase (*PbAGN*), and β -1,3-glucanase (*PbBGN1* and *PbBGN2*), have their expression increased by the *N*-glycosylation inhibition, as detected by qRT-PCR. The observed increases in gene expression levels reveal possible compensatory mechanisms for diminished enzyme activity due to the lack of glycosylation caused by TM.

Keywords: *N*-glycan, *Paracoccidioides brasiliensis*, Cell wall, Fungal cell.

INTRODUCTION

Paracoccidioidomycosis is the most prevalent systemic mycosis in Latin America. This infectious disease is caused by the dimorphic fungus *Paracoccidioides brasiliensis* and *Paracoccidioides lutzii*, following the inhalation of airborne propagules from fungal mycelia. Due to the internal temperature of the host, the propagules become multiple budding yeast forms, resulting in granuloma formation and adversely affecting the functions of infected organs [1-3].

The phase transition implicates variations in the composition of cell wall, a highly dynamic structure that requires remodeling, a process that involves events as cell expansion and division in yeasts, spore germination, hyphal branching, and septum formation in filamentous fungi [4, 5]. The cell wall of *P. brasiliensis* is composed of a network of glycoproteins and polysaccharides; they work in concert to perform essential protective roles for the cell [6], and confer virulence to the fungus. The yeast form of *P. brasiliensis* has

α -glucan in the cell wall, whereas the mycelial form contains large amounts of β -glucan [5, 7]. The distinct composition of cell wall glucans can play important roles in fungal dimorphism and pathogenesis.

Certain *P. brasiliensis* glycolytic enzymes, such as *N*-acetyl- β -D-glucosaminidase, α - and β -glucanase, α -amylase, α -glucan synthase, and β -glucanoyltransferase play important functions with respect to the structure of the cell wall. They are also required for the synthesis, and the remodeling of cell walls in response to environmental changes [8-11]. *N*-glycans are involved in protein folding, intracellular transport, and protection from proteolytic degradation [12]. We previously identified *N*-glycans as essential for fungal growth, morphogenesis, and for the biological activities of some *P. brasiliensis* proteins [[11, 13].

In the current study, we demonstrated that the inhibition of protein *N*-glycosylation affected the expression of genes related to cell wall synthesis. These included *PbGEL3*, *PbFKS1*, and *PbAMY*, which encode the enzymes β -1,3-glucanoyltransferase, 1,3- β -D-glucan synthase, and α -1,4-amylase, respectively. In addition, genes encoding proteins related to cell wall degradation were affected, including *PbNAG1* (*N*-acetyl- β -D-glucosaminidase), *PbAGN* (α -1,3-glucanase), and *PbBGN1* and *PbBGN2* (β -1,3-glucanase).

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The expression levels of these genes, as determined by quantitative polymerase chain reaction (qPCR) assays, were increased following the treatment of *P. brasiliensis* with tunicamycin (TM). Increases in the expression levels of the aforementioned genes could be compensatory mechanisms to overcome the reduced activities of enzymes, due to a lack of glycosylation as a result of the treatment with TM.

MATERIAL AND METHODS

Ethics Statement

This study was approved by the Ethics Committees and the Institutional Review Board of the University of Sao Paulo (approval number 145/2005).

Fungal Strain and Growth Conditions

We used the highly virulent *P. brasiliensis* Pb18 isolate in all experiments. The yeast form was maintained *in vitro* by sub-culturing cells weekly, and maintaining cultures at 36°C in Fava Netto's solid medium containing 1% (w/v) peptone, 0.5% (w/v) yeast extract, 0.3% (w/v) proteose peptone, 0.5% (w/v) beef extract, 0.5% (w/v) NaCl, 4% (w/v) glucose, and 1.2% (w/v) agar at pH 7.2 [14]. To ensure the virulence of Pb18 was maintained, we serially passaged the isolate in BALB/c mice before using in experiments. For yeast growth, cells were incubated in liquid YPD medium containing 2% (w/v) peptone, 1% (w/v) yeast extract, and 2% (w/v) glucose, at 36°C and 100 rpm for 72 h. We prepared TM (Sigma-Aldrich, Saint Louis, MO, USA) in 20 mM NaOH and used it at a final concentration of 15 µg/mL, as previously described [11, 13].

Cell Viability

The viability of fungal cells was verified by staining with fluorescein diacetate and ethidium bromide, as described previously [15]. Only fungal cultures with a viability exceeding 85% were used in our experiments.

Immunoblotting

We performed sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under denaturing conditions (8% and 12% SDS-PAGE), according to the method of Laemmli [16]. We used a Mini-Protean 3 electrophoresis cell for the separation of proteins (Bio-Rad Laboratories, Richmond, CA, USA). Proteins were electrophoresed at 120 V, and gels stained with 0.25% Coomassie Brilliant Blue G-250 (GE Healthcare, Waukesha, WI, USA). Pre-stained standards (Fermentas, Burlington, Ontario, CA) were used for estimating the molecular weight of proteins in each sample. Following electrophoresis, proteins were transferred to Hybond-C Extra nitrocellulose membranes (GE Healthcare). We detected β -1,3-glucanase and 1,3- β -D-glucan synthase by incubating membranes with the relevant specific murine antibodies (1 mg/mL) overnight. Membranes were then incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG (diluted 1:1000 in blocking buffer). The reaction was revealed using the peroxidase substrate ECL (Enhanced Chemo-Luminescence, GE Healthcare).

RNA Isolation, cDNA Synthesis and qPCR Assays

Control and TM-treated yeast were homogenized in *liquid nitrogen* using a small mortar and pestle, and total RNA was isolated using Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. The quality and concentration of RNA samples were assessed by 1.2% (w/v) agarose gel electrophoresis and spectrophotometric analysis. We treated RNA samples with DNase I (Fermentas, Waltham, Massachusetts, USA) to remove genomic DNA, with cDNA subsequently synthesized using a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions. The cDNA samples were diluted 1:25 in water and subjected to qPCR using a CFX96™ Real-Time PCR Detection System (Bio-Rad) and the SsoFast™ EvaGreen® Supermix (Bio-Rad), in accordance with the manufacturer's instructions. The α -tubulin gene was used as an endogenous control for normalization of gene expression levels. The thermal cycling profile used involved an initial denaturation step at 95°C for 10 min, followed by 39 amplification cycles (95°C for 10 s, 60°C for 30 s), and then a melt curve analysis (60–95°C, increasing in 0.5°C increments, for 10 s at each temperature). Gene expression levels were calculated as described previously [17]. The oligonucleotide primers used in the qPCR assays (Table 1) were designed with PerlPrimer v1.1.20.

Prediction of Three-dimensional (3D) Structures and N-glycosylation Sites

The 3D structures of proteins were predicted using the I-TASSER server [18]. Protein Data Bank (PDB) files were uploaded to GLYCAM WEB (www.glycam.crc.uga.edu) to show where *N*-glycans attached at previously predicted sites. We used Man5GlcNAc2 as the pattern oligosaccharide for attaching to structures [19].

Statistical Analysis

Data were analyzed using GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA), and tested by one-way ANOVA (non-parametric) followed by the Bonferroni test, to compare TM-treated yeast samples with controls. Data were the means of representative results from at least three independent experiments performed in triplicate. A *P*-value less than 0.05 were considered statistically significant.

RESULTS

We used TM at 15 µg/mL to treat *P. brasiliensis* as it was previously demonstrated to efficiently inhibit *N*-glycosylation without affecting fungal growth [11, 13]. The expression levels of genes of interest were assessed by qPCR. Prior to the isolation of RNA, cell viability was determined using fluorescein diacetate and ethidium bromide. The relative expression levels of cell wall synthesis-related genes (CWSRG) and cell wall degrading-related genes (CWDRG) were increased in TM-treated yeast cells (Fig. 1A and 1B). The expression levels of all genes we examined were significantly upregulated. In addition, TM treatment resulted in the upregulation of endoplasmic reticulum stress sensors (Fig. 1C).

Table 1. Oligonucleotide primers used in this study, and their target genes.

Protein/enzyme (Approved Gene Symbol)	Oligonucleotide Primer Sequence (5'-3')
β -1,3-glucanase (<i>PbBGN1</i>)	F: GAGAACTGCTACTGTCCACC R: TGGATGGGATTGGACTT TG
β -1,3-glucanase (<i>PbBGN2</i>)	F: CCACAGTCCATTACATCTC R: GTTGAAGACTCAGAGGACAT G
<i>N</i> -acetyl- β -D-glucosaminidase (<i>PbNAG1</i>)	F: TTCTGGATAAGTTGATGGCGG R: AAGGTTTTAGGACGTCTCTGC
α -1,3-glucanase (<i>PbAGN</i>)	F: CAGCAAACACTAAACCCAACG R: CCCTGAACCCACATGTACTAAG
α -amylase (<i>PbAMY</i>)	F: AACTGAATCGTACATCTAGCCG R: GACGCCATTCCGCAATAAAC
1,3- β -D-glucan synthase (<i>PbFKS1</i>)	F: TCTGCGGATTTCATTTTGGG R: GTAGATTGGTGGGCGGATTG
β -1,3-glucanosyltransferase (<i>PbGEL3</i>)	F: CGTTGTCAGCGGAGGTATCGTC R: AGGGCAGGTTCCGAGTTCAGTG
Regulator of the response unfolded protein (<i>PbHACA</i>)	F: GATTCACCCACTCTTGTCCC R: GAATCTGTGAGGTCCAAGTCC
inositol-requiring enzyme 1 (<i>PbIRE1</i>)	F: CACAATTACAGGAGCTTGCG R: GAACCTTGTCTCGTCTAACTC
α -tubulin	F: CGGCTAATGGAAAATACATGGC R: GTCTTGGCCTTGAGAGATGCAA

F, forward primer

R, reverse primer.

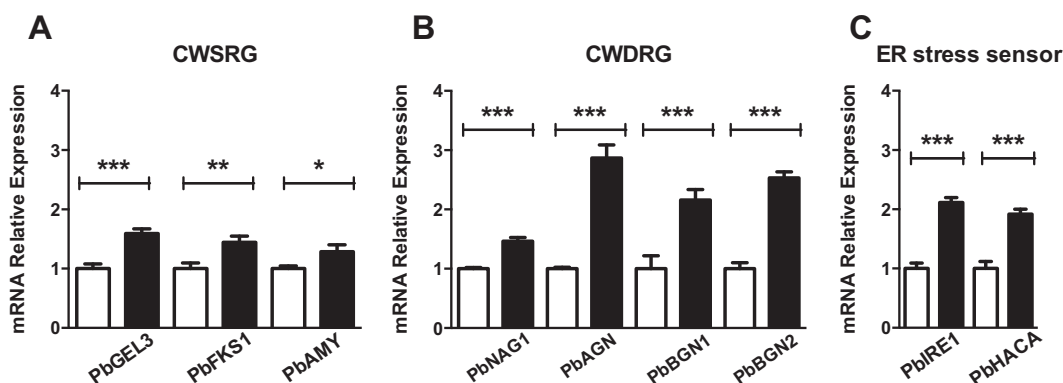


Fig. (1). Expression levels of *PbGEL3*, *PbFKS1*, *PbAMY*, *PbNAG1*, *PbAGN*, *PbBGN1*, *PbBGN2*, *PbIRE1* and *PbHACA* in *P. brasiliensis* yeast cells following treatment with tunicamycin (TM). Control cultures of *P. brasiliensis* were grown in the absence (white bars) of TM, while test cultures were treated with 15 μ g/mL TM (black bars) for 72 h at 37°C. Total RNA was isolated and quantitative polymerase chain reaction (qPCR) assays performed. (A) Cell wall synthesis-related genes (CWSRG). (B) Cell wall degrading-related genes (CWDRG). (C) Endoplasmic reticulum stress sensor-related genes. The $\Delta\Delta$ Ct method was used to calculate the relative amount of RNA transcripts in each sample. The α -tubulin gene was used to normalize expression levels across reactions. Bars corresponds to the standard deviation (SD) of mean values from at least three replicates. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. controls.

To evaluate the importance of *N*-glycans with respect to the production of β -1,3-glucanosyltransferase and 1,3- β -D-glucan synthase, we used immunoblotting to characterize the effects of 15 μ g/mL TM on their production and accumulation. We observed lower levels β -1,3-glucanosyltransferase and 1,3- β -D-glucan synthase for TM-treated samples than those in controls (Fig. 2). These results agree with our previous findings, where the activities of *N*-acetyl- β -D-

glucosaminidase [13] and α -amylase [11] were inhibited in yeast cells following treatment with TM. Our collected data indicate that *N*-glycans are likely required for the accumulation of β -1,3-glucanosyltransferase and 1,3- β -D-glucan synthase.

We verified the influence of *N*-glycosylation on fungal remodeling enzymes using the genome database from the

Broad Institute (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasiliensis/MultiHome.html). We also predicted *N*-glycosylated sequons using the NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc>; Fig. 3). We found that genes *PADG_03695.2* (*PbNAG1*), *PADG_04918.2* (*PbGEL3*), and *PADG_11846.2* (*PbFKS1*) encoded *N*-acetyl- β -D-glucosaminidase (Fig. 3A), β -1,3-glucanosyltransferase (Fig. 3B), and 1,3- β -D-glucan synthase (Fig. 3C), respectively. Prediction of their *N*-glycosylation sites identified three sequons for *N*-glycosylation located at the glycosyl hydrolase domain for *N*-acetyl- β -D-glucosaminidase and β -1,3-glucanosyltransferase (Fig. 4A, 4B). In contrast, two sequons were observed for 1,3- β -D-glucan synthase (Fig. 4C), and these were not located at the glycosyl hydrolase domain of the enzyme. The *PbNAG1* gene comprises 1815 nt, corresponding to 604 amino acids. The *PbGEL3* and *PbFKS1* genes were 1937 and 5854 nt, respectively, and encode proteins of 529 and 1898 amino acids.

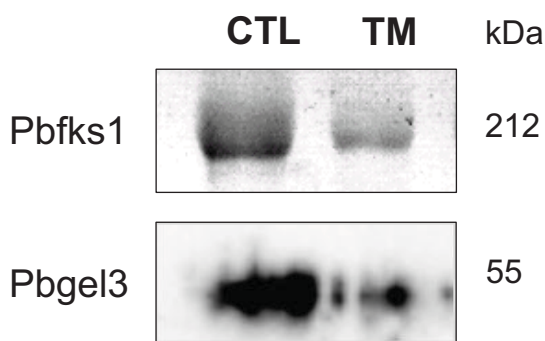


Fig. (2). Immunoblotting analysis of *P. brasiliensis* enzymes related to cell wall remodeling. *P. brasiliensis* yeast cells were grown in the absence (CTL) or presence of 15 μ g/mL TM for 72 h at 37°C. Crude extracts of *P. brasiliensis* cultures were electrophoresed and subjected to immunoblotting. Specific antibodies against β -1,3-glucanosyltransferase (*Pbgel3*) and 1,3- β -D-glucan synthase (*Pbfks1*) were used to detect these proteins by chemoluminescent substrate for peroxidase.

We sought to predict the 3D structures of these enzymes (Fig. 4 A-C) using the I-TASSER server [18]. Putative glycans were inserted into the predicted *N*-glycosylation sites of each protein. The obtained 3D structural models of *N*-acetyl- β -D-glucosaminidase, β -1,3-glucanosyltransferase, and 1,3- β -D-glucan synthase showed that their glycosyl hydrolase domains were composed of α -helices and β -sheets.

DISCUSSION

Our findings show that inhibition of *N*-linked glycosylation, *via* treatment with 15 μ g/mL TM, resulted in an increase in the expression levels of genes related to cell wall remodeling in *P. brasiliensis* yeast cells. Treatment with TM also resulted in decreased production of β -1,3-glucanosyltransferase and 1,3- β -D-glucan synthase. These findings reflect those we have presented previously, where the enzymatic activities of *N*-acetyl- β -D-glucosaminidase and α -amylase were inhibited when yeast cells were treated with TM [11, 13]. Our results suggest that these enzymes are

N-glycan dependent to be secreted and or to exert satisfactory enzyme activity.

It has been shown that *N*-linked glycans can be inhibited by many substances, such as TM. This nucleoside antibiotic adversely affects *N*-glycosylation, and is widely used to block this particular post-translational modification of proteins. TM exerts its effects by hindering the initial transfer of uridine diphosphate-*N*-acetyl-glucosamine to dolichol-phosphate [20, 21]. We analyzed the effects of TM upon endoplasmic reticulum stress sensors in yeast (Fig. 1C). We observed increased expression levels of *PbHACA* and *PbIRE1*, which have been described as activators of the cellular response to endoplasmic reticulum-based stress signals [22, 23].

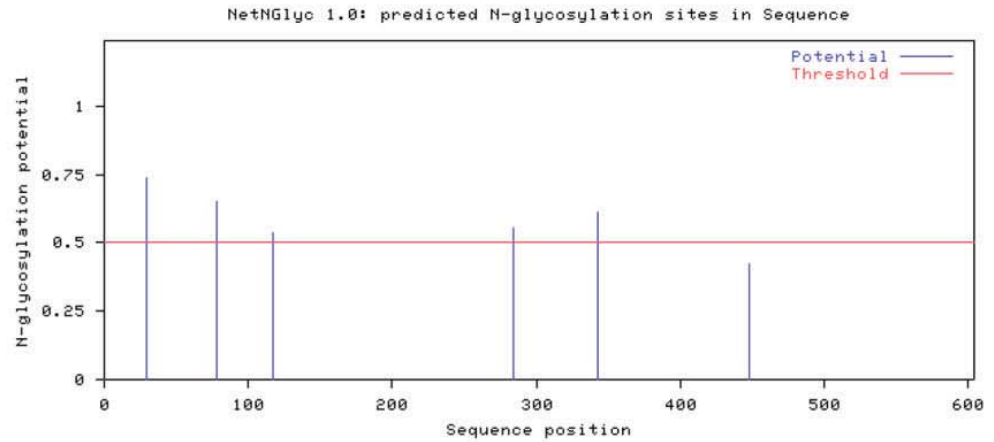
We previously observed intracellular accumulation of paracoccin, a glycoprotein with *N*-acetyl- β -D-glucosaminidase activity [10]. In addition, we found that treatment of yeast cells with TM resulted in lower enzymatic activities for paracoccin and α -1,4-amylase [11, 13]. Therefore, we hypothesized that higher expression levels of genes related to cell wall remodeling in TM-treated *P. brasiliensis* yeast cells could be an attempt, at the transcriptional level, to compensate for reduced enzymatic activity. The enzymes encoded by *CWSRG* and *CWDRG* play important roles related to the synthesis and degradation of the cell wall, which is mainly composed of chitin and glucan [4, 5, 7]. Therefore, altered expression levels of these genes might affect synthesis or degradation of the cell wall, which would likely be crucial for the dimorphism of the fungus.

We predicted the 3D structure of the enzymes investigated in this study. However, the absence of *P. brasiliensis*-related protein structures in the PDB meant it was not possible to generate high homology models. The reduction in *N*-acetyl- β -D-glucosaminidase activity strongly suggests that the presence of *N*-glycans, at three sites in the enzymatic domain, probably interferes with its performance. Similar results were obtained for β -1,3-glucanosyltransferase, which also contained three predictable *N*-glycosylation sites at the enzymatic domain. Our findings lead us to believe that β -1,3-glucanosyltransferase activity is also *N*-glycan dependent. In contrast, the predicted structure of 1,3- β -D-glucan synthase revealed two sequons for *N*-glycosylation, and these were not located at the enzymatic domain. However, levels of this enzyme were also reduced in TM-treated yeasts, a fact that makes mandatory further studies on the activity of 1,3- β -D-glucan synthase from *P. brasiliensis*.

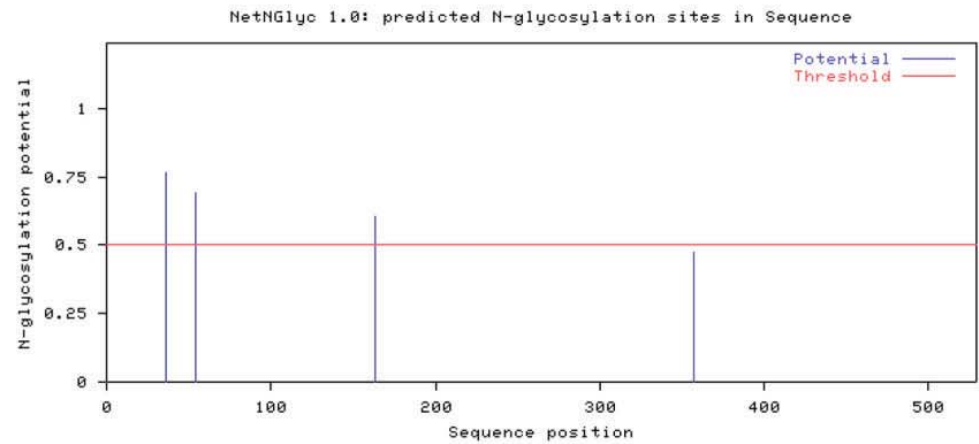
The *N*-glycosylation of proteins is crucial for a wide range of biological processes. The removal, and insertion of putative *N*-glycan sites on proteins leads to drastic effects upon cellular organization. The underglycosylation of enzymes often leads to reduced activity, and altered substrate specificity [24], a fact that highlights the diverse influence exerted by individual *N*-glycans on the properties of proteins [24]. Fungal *N*-glycans are important for growth, morphogenesis, phase transitions, and the mediation of interactions with host cells [11, 13, 25]. Taken together, our results show that at a transcriptional level of *P. brasiliensis* glycobiology may contribute for the elucidation of the fungal biology and the pathogenesis of fungal infections.

A

PADG_03695.2
PbNAG1

**B**

PADG_04918.2
PbGEL3

**C**

PADG_11846.2
PbFKS1

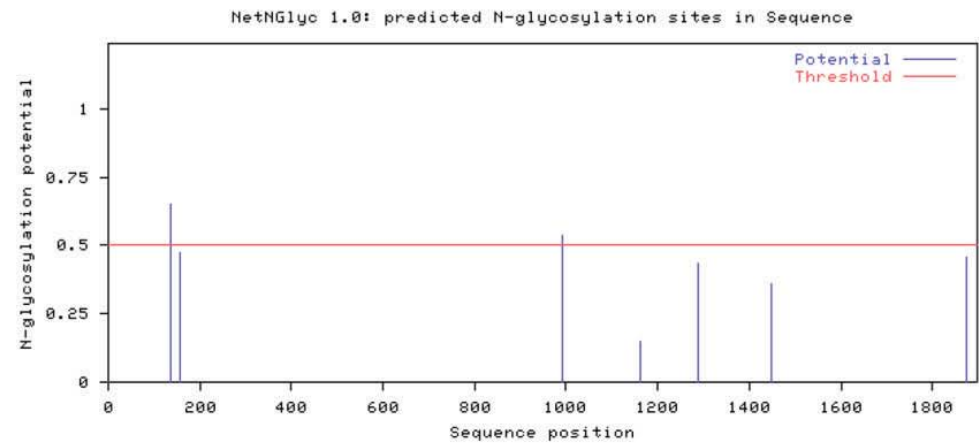


Fig. (3). Predicted *N*-glycan sites of *P. brasiliensis* *N*-acetyl- β -D-glucosaminidase, β -1,3-glucanosyltransferase, and 1,3- β -D-glucan synthase. (A) *N*-acetyl- β -D-glucosaminidase, encoded by *PbNAG1*. (B) β -1,3-glucanosyltransferase, encoded by *PbGEL3*. (C) 1,3- β -D-glucan synthase, encoded by *PbFKS1*. Sequences were uploaded to the NetNGlyc 1.0 Server (<http://www.cbs.dtu.dk/services/NetNGlyc>) and submitted for analysis. Potential sites (vertical lines) crossing the threshold (horizontal lines at 0.5) are predicted to be glycosylated.

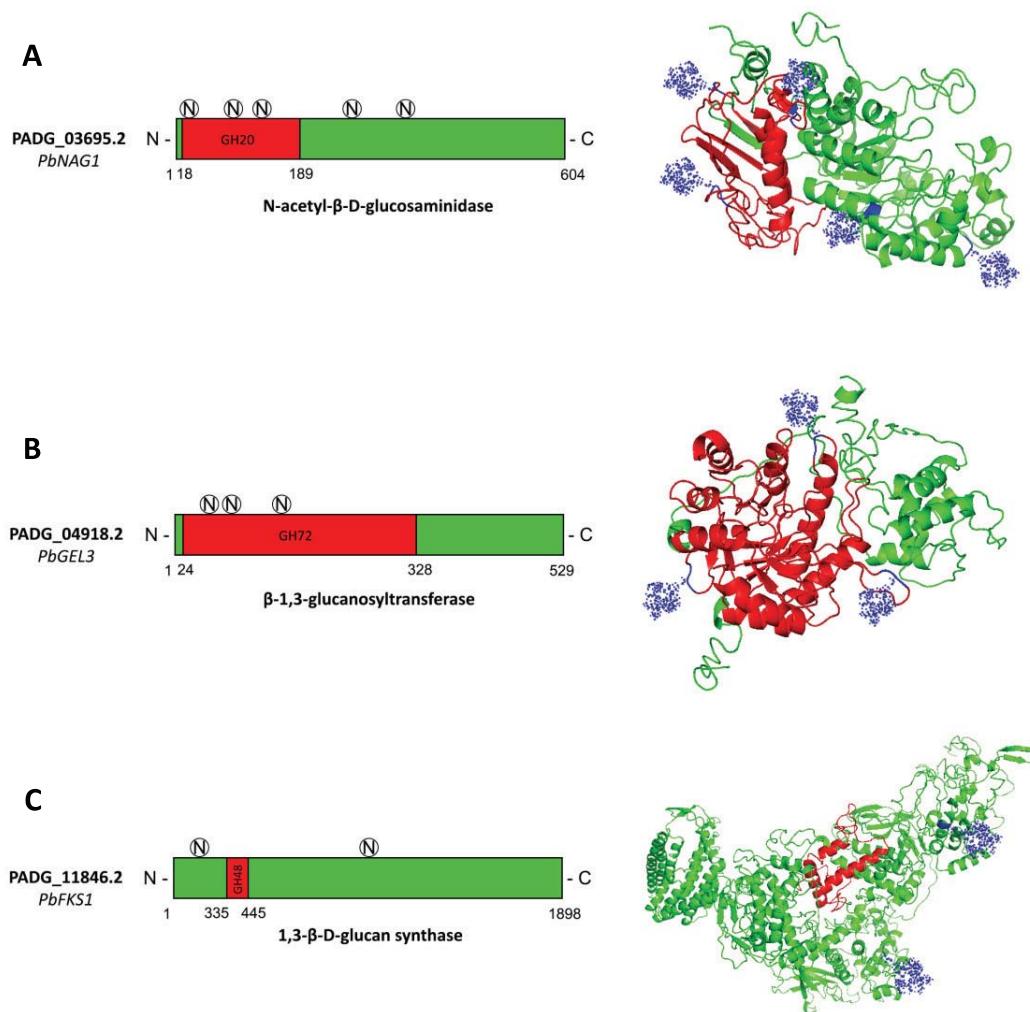


Fig. (4). Predicted 3D structures of *P. brasiliensis* enzymes related to cell wall remodeling. **(A)** *N*-acetyl- β -D-glucosaminidase. **(B)** β -1,3-glucanosyltransferase. **(C)** 1,3- β -D-glucan synthase. Domains for the glycosyl hydrolase (GH) family and *N*-glycosylation positions are highlighted in red and blue, respectively. Circled letters (N) represent predicted *N*-glycosylation sites. The 3D structures of these enzymes were predicted using I-TASSER and NetNGlyc 1.0 server.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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