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Research article

# Purification and biochemical characterization of a novel thermophilic exo-β-1,3-glucanase from the thermophile biomass-degrading fungus *Thielavia terrestris* Co3Bag1

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ABSTRACT

*Background:* The aim of this work was to purify and characterize exo-β-1,3-glucanase, namely, *Tt*BgnA, from the thermophilic fungus *Thielavia terrestris* Co3Bag1 and to identify the purified enzyme.
*Results:* The thermophilic biomass-degrading fungus *T. terrestris* Co3Bag1 displayed β-1,3-glucanase activity when grown on 1% glucose. An exo-β-1,3-glucanase, with an estimated molecular mass of 129 kDa, named *Tt*BgnA, was purified from culture filtrates from *T. terrestris* Co3Bag1. The enzyme exhibited optimum activity at pH 6.0 and 70°C and half-lives ($t_{1/2}$) of 54 and 37 min at 50 and 60°C, respectively. Substrate specificity analysis showed that laminarin was the best substrate studied for *Tt*BgnA. When laminarin was used as the substrate, the apparent $K_M$ and $V_{max}$ values were determined to be 2.2 mg $mL^{-1}$ and 10.8 U/mg, respectively. Analysis of hydrolysis products by thin-layer chromatography (TLC) revealed that *Tt*BgnA displays an exo mode of action. Additionally, the enzyme was partially sequenced by tandem mass spectrometry (MS/MS), and the results suggested that *Tt*BgnA from *T. terrestris* Co3Bag1 could be classified as a member of the GH-31 family.
*Conclusions:* This report thus describes the purification and characterization of *Tt*BgnA, a novel exo-β-1,3-glucanase of the GH-31 family from the thermophilic fungus *T. terrestris* Co3Bag1. Based on the biochemical properties displayed by *Tt*BgnA, the enzyme could be considered as a candidate for potential biotechnological applications.
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## 1. Introduction

β-1,3-Glucans are normally found in the cell wall of fungi [1,2,3], in the amorphous layer of the plant cell wall, and in bran cereal grains [4]. Exo-β-1,3-glucanases (E.C.3.2.1.58) catalyze the release of glucose from the nonreducing ends of β-1,3-glucans [5,6]. Exo-β-1,3-glucanases have been classified mainly in the glycosyl hydrolase families GH3, GH5, GH17, and GH55 [7]. Nevertheless, to the best of our knowledge, thus far, there are no reports of exo-β-1,3-glucanases classified as members of the GH31 family.

β-1,3-Glucanases are of high importance because of their potential application in the biological control of fungal pathogens in crops [5,8,9,10]. In the pharmaceutical industry, β-1,3-glucanases have been suggested to synthesize drug carriers [11], whereas in the medical field, products of β-1,3-glucanases have been proven as immunomodulatory anticancer drugs *in vivo* [12]. Moreover, the simple sugars released by the action of β-glucanase activity have been used as raw material in the production of biofuels such as bioethanol [13]. In the food industry, β-1,3-glucanases have been proposed for the production of beer [14], whereas in the wine industry, they have been used to improve organoleptic properties [15,16]. Additionally, β-glucanases have been of interest to improve the production of feed and animal nutrition [17].

Exo-β-1,3-glucanases are mainly produced by fungi, *e.g., Trichoderma harzianum* [18], *Phanerochaete chrysosporium* [19], *Talaromyces emersonii* [3], and *Volvariella volvacea* [20]. Fungal β-1,3-glucanases seem to fulfill different functions. Apparently, they play a physiological role in morphological processes during fungal extension and differentiation [7,10,21]. Researchers have also pointed out the role of β-1,3-glucanases in the mobilization of β-glucans under

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conditions where the carbon source has been exhausted and as autolytic enzymes in fungus [7,10,22,23,24]. Additionally, an increase in the glucanase activity, upon entering the stationary phase of growth, has been reported for members of the Fungi kingdom when grown with glucose as the carbon source, *e.g., T. viride* [25], *Botryosphaeria rhodina* [26], and *Kluyveromyces marxianus* [27]. Fungal β-1,3-glucanases have also been associated with pathogen–plant interactions, and they degrade β-1,3-glucan in vascular tissues of the host [10,28].

The ascomycete fungus *Thielavia terrestris* is of interest, as it breaks down lignocellulosic biomass and is a source of thermostable enzymes [29]. The genome sequence of *T. terrestris* NRRL 8126 has been reported and compared to that of *Myceliophthora thermophila* ATCC 42464 [30]. The thermophilic biomass-degrading fungus *T. terrestris* Co3Bag1 was isolated from sugarcane bagasse compost, and the purification and characterization of a hyperthermophilic and thermostable β-1,4-xylanase (*Tt*XynA) has been recently reported by our research group [31]. However, to the best of our knowledge, there are no reports describing the biochemical properties of the exo-β-1,3-glucanase from members belonging to the genus *Thielavia*.

In this study, we report the purification and biochemical characterization of an exo-β-1,3-glucanase from the thermophilic fungus *T. terrestris* Co3Bag1 and the identification of the purified enzyme by tandem mass spectrometry (MS/MS).

## 2. Materials and methods

### 2.1. Strains and culture conditions

*T. terrestris* Co3Bag1 (accession number CDBB-H-1938) was obtained from the Colección Nacional de Cepas Microbianas y Cultivos Celulares (CINVESTAV-IPN, México), and the fungus was maintained at 4°C on malt extract agar [32].

Growth kinetics and exo-β-1,3-glucanase activity measurements were carried out by inoculating $1 \times 10^6$ spores in 500 mL Erlenmeyer flask with 200 mL of basal medium previously described by Zouari-Mechichi et al. [33]. The basal medium contained 10 g/L glucose, 5 g/L peptone, 1 g/L yeast extract, 2 g/L ammonium tartrate, 1 g/L $KH_2PO_4$, 0.5 g/L KCl, and 1 mL of solution of trace elements. The fungus *T. terrestris* Co3Bag1 was also cultured in the medium described by Rapp [22] containing 3% (*w*/*v*) glucose as the carbon source and in the medium described by Li et al. [34] containing 4% (w/v) wheat bran as the carbon source. Flasks were incubated at 45°C and shaken at 120 rpm for 15 days. Every 24 h, each flask was assayed for biomass (dry weight) and β-1,3-glucanase activity.

### 2.2. Enzymatic assay and protein determination

β-1,3-Glucanase activity was determined by measuring the amount of reducing sugar released from the hydrolysis of laminarin (Sigma Chemical), used as the substrate for β-1,3-glucanase activity, unless otherwise stated. Initial standard assay mixture (1 mL) contained 860 μL of 50 mM $Na_2HPO_4$-citric buffer (pH 6.0), 100 μL of 2% laminarin, and 40 μL of enzyme preparation. For the choice of initial conditions for enzymatic assay, an enzyme progress curve against varying time, temperature, enzyme concentration, and substrate concentration was independently constructed. The reaction was determined by immersing the samples in a water bath at 70°C for 5 min and monitoring the reducing sugar content by the colorimetric method 3,5-dinitrosalicylic acid (DNS) [35] using D-glucose as the calibration standard. Enzyme and substrate blanks were also included. The results presented are expressed as mean ± standard deviation of two independent experiments conducted in triplicate. One unit of β-1,3-glucanase activity was defined as the amount of enzyme that released 1 μmol of glucose per min from laminarin under the described conditions. The protein concentration was measured by the Lowry method [36], using bovine serum albumin (Life Technologies, Grand Island, NY, USA) as the standard.

### 2.3. Purification of the enzyme

For exo-β-1,3-glucanase production, a total of eighteen Erlenmeyer flasks (500 mL) were used as described by Zouari-Mechichi [33], and each flask was inoculated with $1 \times 10^6$ spores. Cultures were incubated at 45°C for six days under orbital agitation at 120 rpm. The mycelium was removed by vacuum filtration, and the culture supernatant, named crude extract, was used as a source of exo-β-1,3-glucanase activity. Subsequently, *T. terrestris* crude extract (3.5 L) was concentrated by ultrafiltration at 4°C using a polyethersulfone membrane with a cutoff weight of 30,000 Da (PALL, NY, USA). When a 350-fold concentration was reached, the ultrafiltrate was dialyzed overnight at 4°C against buffer A (50 mM L-histidine buffer pH 6.0, 25 mM KCl, 0.1 mM phenyl methyl sulfonyl fluoride (PMSF), and 5% (*v*/v) glycerol). Then, 15 mL of the dialysate was loaded onto a UNOsphere™ Q (Bio-Rad) column (15 × 1.5 cm), with a packaging volume of 17 mL, which had been previously equilibrated with buffer A.

Adsorbed proteins were eluted with a linear gradient of KCl (0.025–1 M) in buffer A at a constant flow rate of 2 mL/min. Fractions with β-1,3-glucanase activity were pooled and concentrated to 2 mL by filtration through a Microsep™ Advance Centrifugal Device (MWCO 30K) PALL ®. The ultrafiltrate was then dialyzed overnight at 4°C against $Na_2HPO_4$-citric buffer (50 mM, pH 6.0, 25 mM KCl, 0.1 mM PMSF, and 5% (*v*/v) glycerol). A sample (2.5 mL) of dialysate was loaded onto and applied to Bio-Gel® P-100 Gel column (2.5 × 22 cm), previously equilibrated with 50 mM $Na_2HPO_4$-citric acid buffer (pH 6.0), and fractions were eluted at a flow rate of 0.05 mL/min. The volume of the fractions was 1.1 mL. Fractions with β-1,3-glucanase activity were pooled and analyzed by 10% sodium dodecyl sulfate (SDS)-PAGE.

### 2.4. SDS-PAGE analysis

The purity of the enzyme was confirmed by 10% SDS-PAGE performed on a vertical slab gel as described by Laemmli [37]. Proteins were stained with 0.05% Coomassie ® Brilliant Blue R-250. The molecular weight of the proteins was estimated with reference to a broad range of molecular weight protein standards (Bio-Rad). Gels were recorded and analyzed using a gel documentation system (DigiDoc-It Imaging System, UVP).

### 2.5. Zymogram analysis

Enzymatic activities in the gel were performed using a modified version of the method previously described by Pan et al. [38], with some modifications as follows. Briefly, protein samples were separated in 10% polyacrylamide gel copolymerized with 0.5% of laminarin under semi-denaturing conditions. Protein samples were resuspended in SDS sample buffer without β-mercaptoethanol, and the samples were incubated at 37°C for 30min. After electrophoresis, gels were washed with 1% Triton X-100 and 25% isopropanol in 50 mM $Na_2HPO_4$-citric buffer (pH 6.0) for 2 h with gentle shaking to remove the SDS; the gels were equilibrated with fresh buffer every 30 min. Gels were washed with distilled water and then incubated in 50 mM $Na_2HPO_4$-citric acid buffer, pH 6.0 at 50°C for 120 min. Bands with β-1,3-glucanase activity were visualized after heating the gel with 2,3,5-triphenyl tetrazolium chloride solution.

### 2.6. Biochemical characterization

To study the biochemical properties of *Tt*BgnA, purified preparations of the enzyme were used. Before all determinations (*e.g.*, optimal pH and temperature, pH stability, thermal stability, kinetic parameters ($K_M$ and $V_{max}$), effect of metal ions, and substrate specificity), enzyme progress curves against varying time and protein concentration were constructed to determine the correct time for β-1,3-glucanase assay

and protein concentration for linearity. This protocol was usually performed to confirm a linear relationship between time or protein concentration *versus* β-1,3-glucanase activity.

### 2.7. Optimal pH and pH stability

The effect of pH on enzymatic activity was determined by varying the pH of the reaction mixtures using 50 mM $Na_2HPO_4$-citric acid buffer (pH 4.0–7.0), 50 mM citrate buffer (pH 4.0–6.5), 50 mM buffer acetate (pH 3.5–5.5), and 50 mM phosphate buffer (pH 5.5–8.0). Reaction mixtures were incubated at 70°C for 5 min. For pH stability assays, samples of the purified enzyme were incubated, without substrate, at 25°C for 2 h at different pH values ranging from 2.5 to 7.5. Then, the residual β-1,3-glucanase activity was measured under standard conditions (70°C at pH 6.0 for 5 min). Activity was estimated as a percentage of the maximum, which was taken as 100%.

### 2.8. Optimal temperature and thermal stability

The effect of temperature on enzymatic activity was estimated by conducting the activity assay at different temperatures ranging from 25 to 85°C in 50 mM $Na_2HPO_4$-citric acid buffer, pH 6.0. Reaction mixtures were incubated for 5 min under the conditions described. The thermal stability of the enzyme was investigated after preincubation at 50, 60, 65, 70, and 80°C without substrate. Residual enzyme activities at specific time points were determined under standard conditions (70°C, pH 6.0, and 5 min). To determine the $t_{½}$ at 50, 60, 65, 70, and 80°C, the logarithm of percent remaining activity at the temperature tested was plotted *versus* time, as described by Atkins and de Paula [39]. The inactivation rate constants were calculated using the OriginLab program. Activity was estimated as a percentage of the maximum, which was taken as 100%.

### 2.9. Effect of metal ions

The effect of metal ions on the β-1,3-glucanase activity was evaluated in the presence of various metal ions ($CaCl_2$, CsCl, $CuCl_2$, $FeCl_3$, KCl, LiCl, $MnCl_2$, NaCl and $NiCl_2$) at concentrations of 1 and 5 mM. Enzymatic activity was determined under standard conditions. Enzymatic activity was expressed as a percentage of the activity observed in the absence of any metal ion (control), which was taken as 100%.

### 2.10. Substrate specificity and kinetic parameters

Substrate specificity of enzyme was determined under optimal assay conditions (70°C, pH 6.0) using 2 mg/mL of laminarin, gentiobiose, curdlan (β-1,3-glucan from *Euglena gracilis*), cellobiose, starch, glycogen, amylose, maltose, saccharose, methyl-α-D-glucopyranoside, or methyl-α-D-mannopyranoside (Sigma-Aldrich, USA) as the substrate. Activity was estimated as percentage of enzymatic activity obtained with laminarin as the substrate, which was taken as 100%. The effect of the concentration of laminarin at concentrations ranging from 0.5 to 12 mg/mL was evaluated under optimal assay conditions (70°C at pH 6.0 for 5 min). The apparent $K_M$ and $V_{max}$ values were determined by a nonlinear regression method using least squares (OrigingLab program).

### 2.11. Analysis of hydrolysis products

To analyze the hydrolysis products released from laminarin by the action of *Tt*BgnA, a reaction mixture containing *Tt*BgnA (5 U) and 1% laminarin in 50 mM $Na_2HPO_4$-citric acid buffer, pH 6.0 was incubated at 40°C for 48 h. Additionally, a test using 4-nitrophenyl β-D-glucopyranoside (*p*NPG) or saccharose as the substrate was carried out under the same conditions. Aliquots were withdrawn at different times, and hydrolysis products were then analyzed by TLC. A total of 3 μL of each sample and 2 μL of standard (*p*NPG, laminarin, glucose, gentiobiose, and cellobiose) were spotted on a silica gel (Merck KGaA, Darmstadt, Germany). The hydrolysis products were separated in a solvent system consisting of ethanol (30% *v*/v), butanol (50% v/v), and $H_2O$ (20% v/v). The plate was then sprayed with sulfuric acid (15% v/v) and heated at 80°C for 40 min in an oven to reveal the products.

### 2.12. Partial amino acid sequencing and sequence analysis

Purified *Tt*BgnA was partially sequenced by tandem mass spectrometry (MS/MS) at the Unidad de Proteómica, Instituto Nacional de Medicina Genómica (INMEGEN, México) and Laboratorio Nacional de Servicios Experimentales (LaNSE, CINVESTAV). The amino acid sequence of peptides obtained was analyzed for confiability using the Paragon algorithm in ProteinPilot™ software, the UniProt Knowledgebase database, and other tools available at NCBI (https://www.ncbi.nlm.nih.gov/) and Expasy (https://www.expasy.org/).

The sequence of the GH31 protein from *T. terrestris* NRRL 8126 (UniProtKB accession number G2RFM7) was also used as a query to retrieve sequences from the NCBI database. Afterwards, multiple sequence alignment was performed using ESPript 3 (http://espript.ibcp.fr/ESPript/ESPript/) [40] to determine the elements of the secondary structure.

### 2.13. Phylogenetic reconstruction

Phylogenetic inference of the β-glucanase from *T. terrestris* NRRL 8126 was made with other β-glucanases of the GH31 family and with exo-β-1,3-glucanases of GH3, GH5, GH17, and GH55 families. The amino acid sequences were obtained from UniProtKB and NCBI databases. A multiple sequence alignment of the sequences was conducted by MUSCLE with MEGA v 7.0.26 [41] using default parameters. A phylogenetic reconstruction analysis was performed by using the maximum likelihood (ML) statistical method based on the Poisson correction model [41]. The test to assess the phylogeny used was performed by the Bootstrap method with 1000 bootstrap replications. The initial tree for the heuristic search was obtained automatically by applying the neighbor-joining and BIONJ algorithms to a matrix of pairwise distances estimated using a JTT model and then selecting the topology with a superior log likelihood value. As outgroup sequence for this analysis, we used *Aspergillus niger* (β-glucosidase GH1 family: accession number from UniProtKB: Q9P456).

## 3. Results

### 3.1. Exo-β-1,3-glucanase activity

At the beginning of this work, the ability of *T. terrestris* Co3Bag1 to produce exo-β-1,3-glucanase was evaluated by growing this fungus in three different growth media containing 1% glucose, 3% glucose, or 4% wheat bran as the carbon source, at 45°C for 15 days. Exo-β-1,3-glucanase activity was detected throughout fermentation. The fungus produced the highest β-1,3-glucanase activity (0.71 U/mL) after 6 days of growth (Fig. 1a) in the culture with 1% glucose. On the 6th day of fermentation, the stationary phase from *T. terrestris* Co3Bag1 began and the concentration of the carbon source fell to a low level (Fig. 1a). On the other hand, the highest β-1,3-glucanase activity of 0.14 and 0.80U/mL was observed for the culture with 3% glucose and 4% wheat bran, respectively, after 6 days of growth in both cases (Fig. 1b, c).

### 3.2. Purification of TtBgnA

A β-1,3-glucanase was purified from the culture supernatant of *T. terrestris* Co3Bag1 grown on 1% glucose as the only carbon source. The

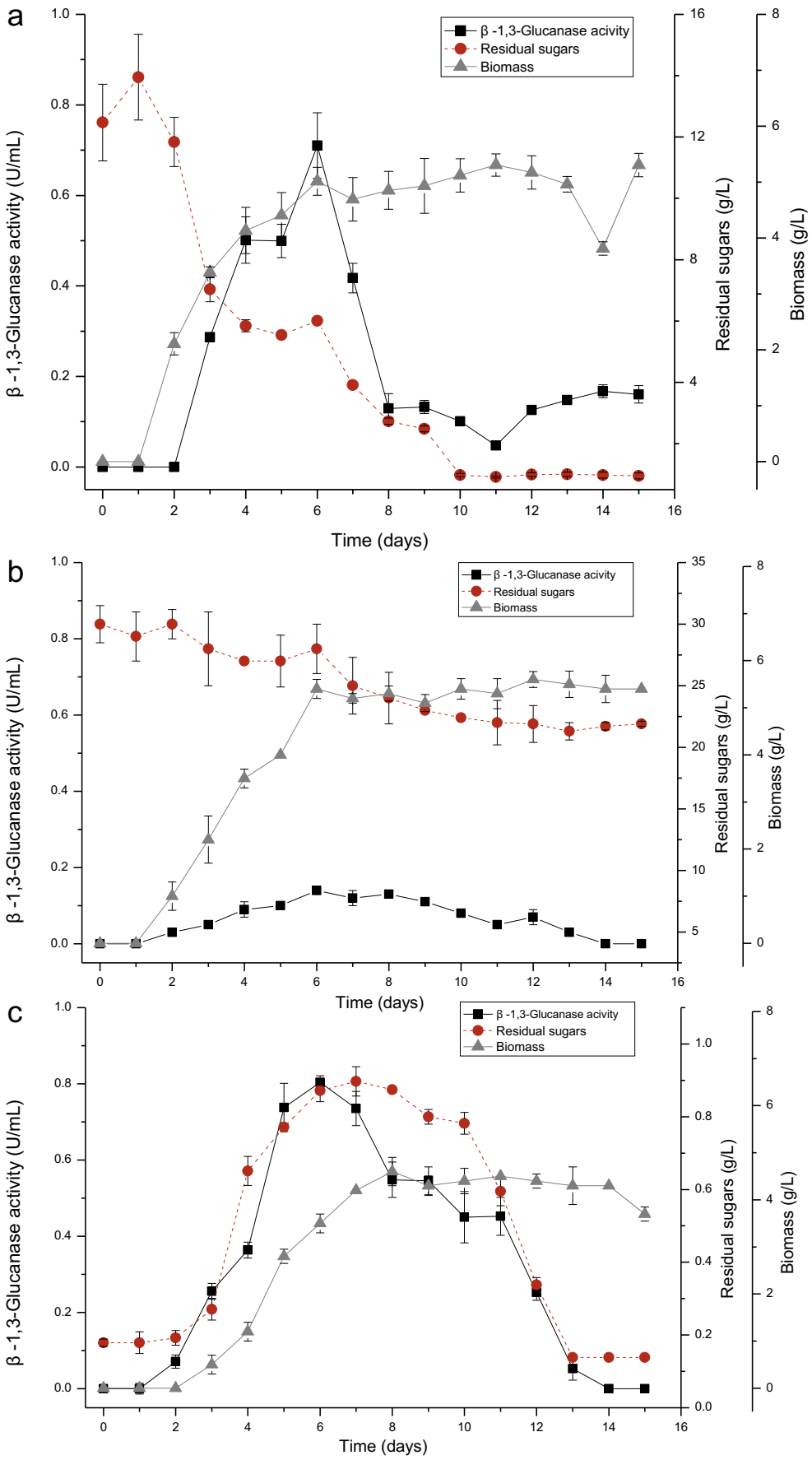


**Fig. 1.** Growth, β-1,3-glucanase activity, and residual sugars in the culture medium of *T. terrestris* Co3Bag1. Growth was monitored by cell dry weight, and β-1,3-glucanase activity and residual sugars were assessed by the DNS method. (a) Fermentation with 1% (*w*/*v*) glucose; (b) Fermentation with 3% (w/v) glucose; and (c) Fermentation with 4% (w/v) wheat bran, as carbon sources.

**Table 1**
Summary of purification of *Tt*BgnA.

| Step | Total activity (U) | Total protein (mg) | Specific activity (U/mg) | Yield (%) | Fold purification |
|---|---|---|---|---|---|
| Crude extract | 138.02 | 1641.23 | 0.08 | 100.00 | 1.00 |
| Ultrafiltration (3.0 kDa) | 80.12 | 120.40 | 0.67 | 58.05 | 8.38 |
| AEC[a] | 10.51 | 10.10 | 1.04 | 7.61 | 13.00 |
| GFC[b] | 0.84 | 0.14 | 6.00 | 0.61 | 75.00 |

[a] AEC: Anion Exchange Chromatography.
[b] GFC: Gel Filtration Chromatography.

culture supernatant was concentrated by ultrafiltration, and then the enzyme was purified by anion exchange and gel filtration chromatography; a summary of these purification steps is shown in Table 1. The β-1,3-glucanase was purified to homogeneity with a 75-fold purification factor, a recovery yield of 0.6%, and specific activity of 6 U/mg. SDS-PAGE analysis of the purified enzyme showed a single band with an estimated molecular mass of 129 kDa (Fig. 2a), and this band displayed β-1,3-glucanase by zymogram analysis (Fig. 2b). Then, the β-1,3-glucanase of 129 kDa was named *Tt*BgnA.

### 3.3. Biochemical characterization of TtBgnA

#### 3.3.1. Optimal pH and pH stability

*Tt*BgnA showed maximal activity at pH 6.0 and exhibited approximately 60% of its maximal activity at different pH values ranging from 4.5 to 7.0 (Fig. 3a). pH stability assays showed that *Tt*BgnA retained more than 80% of its activity in the pH range from 5.0 to 6.5 after 2 h 30 min at 25°C and retained more than 50% activity from pH 4.5 to 7.0 (Fig. 3b).

#### 3.3.2. Optimal temperature and thermal stability

The enzyme showed optimal activity at 70°C and pH 6.0 and displayed more than 50% of its maximum activity at a temperature that ranged from 55 to 75°C (Fig. 4a). An increase in exo-β-1,3-glucanase activity was observed as the temperature increased in the range of 40 to 70°C. The kinetics were of first order with a coefficient of determination between 0.97 and 0.99. *Tt*BgnA showed low stability at 70°C ($t_{½}$ 13 min) with an inactivation rate constant ($k$) of 0.0521 $min^{-1}$; whereas at 80°C ($t_{½}$ 2 min), a $k$ of 0.2874 $min^{-1}$ was observed. The enzyme was more stable at 50, 60, and 65°C with $t_{1/2}$ values of 54, 37, and 18 min, respectively (Fig. 4b). The rate constant of thermal inactivation at 50, 60, and 65°C was 0.0129, 0.0187, and 0.0379 $min^{-1}$, respectively.

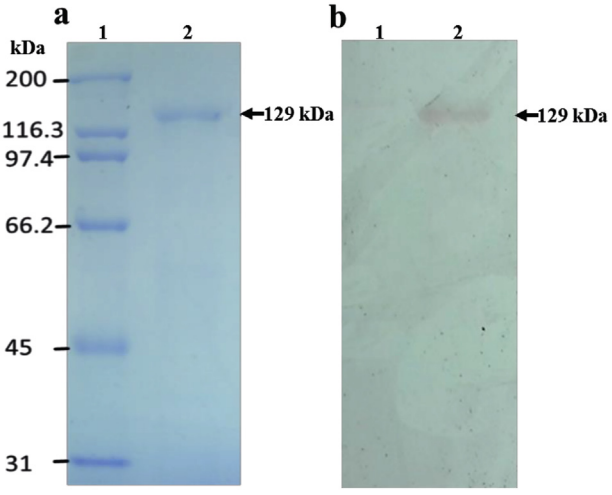


**Fig. 2.** (a) SDS-PAGE analysis of *Tt*BgnA. Lane (1) molecular weight (MW) protein standards, lane (2) *Tt*BgnA. (b) Zymogram analysis of β-1,3-glucanase activity. Lane (1) MW protein standards (negative control), lane (2) *Tt*BgnA. A total amount of 5.2 μg of purified *Tt*BgnA was loaded in each gel.

#### 3.3.3. Effect of metal ions on enzyme activity

The effect of several metal ions ($Ca^{2+}$, $Cs^{1+}$, $Cu^{2+}$, $Fe^{3+}$, $K^{1+}$, $Li^{1+}$, $Mn^{2+}$, $Na^{1+}$, and $Ni^{2+}$) on *Tt*BgnA activity was determined at a final concentration of 1 and 5 mM each (Table 2). The activity of *Tt*BgnA increased to 200% and 167% in the presence of $Li^{1+}$ (5 mM) and $Mn^{2+}$ (1 mM), respectively, whereas no activity was detected at a higher concentration of $Mn^{2+}$ (5 mM). $Ca^{2+}$, $Cu^{2+}$, and $Na^{1+}$ had no apparent effect on the enzymatic activity of *Tt*BgnA at the concentrations tested (Table 2).

#### 3.3.4. Substrate specificity and kinetic parameters

As shown in Table 3, *Tt*BgnA showed remarkably higher activity toward laminarin (β-1,3/β-1,6 link) than toward gentiobiose (β-1,6-glucan link), amylose (α-1,4 link), and saccharose (α-1,4 link), whereas no activity was detected on cellobiose (β-1,4 link), starch, and glycogen (α-1,4/α-1,6 links). The kinetic parameters of *Tt*BgnA were assayed under optimal pH and temperature conditions using laminarin as the substrate. For laminarin, the $K_M$ was 2.2 mg $mL^{-1}$ and the $V_{max}$ was 10.8 U/mg (Fig. 5).

#### 3.3.5. Hydrolytic action

The mode of action of *Tt*BgnA toward laminarin (β-1,3/β-1,6 link) was analyzed by the production of reducing sugar at different times and the hydrolysis products by silica gel thin-layer chromatography (Fig. 6a). Incubation of laminarin with the enzyme resulted in the production of gentiobiose (β-1,6-glucan link) and glucose, the final products of hydrolysis. When the enzyme was incubated with *p*NPG, the hydrolysis product detected was glucose (Fig. 6b), whereas no hydrolysis products were detected with saccharose as the substrate (Fig. S1).

### 3.4. TtBgnA partial sequencing and sequence analysis of the GH31 protein from T. terrestris NRRL 8126

Purified *Tt*BgnA was identified by partial amino acid sequencing. Bioinformatics analysis corresponding to amino acid sequences of the 18 peptides obtained from *Tt*BgnA showed 85–100% identity (Table 4) to the GH31 protein from *T. terrestris* NRRL 8126 (UniProtKB accession number G2RFM7) (Fig. 7). Furthermore, peptide (3) showed 95% identity with α-glucosidase from *Madurella mycetomatis* (UniprotKB accession number A0A175VX48), while peptide (4) showed 79% identity with the GH31 protein from the fungus *Myceliophthora thermophila* (Accession number G2Q431). Interestingly, peptides (4, 5, and 6) were associated by similarity to the Glycosyl hydrolase 31 family domain (http://www.ebi.ac.uk/interpro/entry/IPR011013).

Similarity analysis of the amino acid sequence for the GH31 protein from *T. terrestris* NRRL 8126 (UniProtKB accession number G2RFM7) revealed 75.4% identity to the GH31 protein belonging to the GH31 family from *M. thermophile* ATCC 42464 (G2Q431), 71.9% identity to α-glucosidase belonging to the GH31 family from *M. mycetomatis* (A0A175VX48), 70.9% identity to α-glucosidase belonging to the GH31 family from *Neurospora crassa* ATCC 24698 (V5IP80), 69.8%

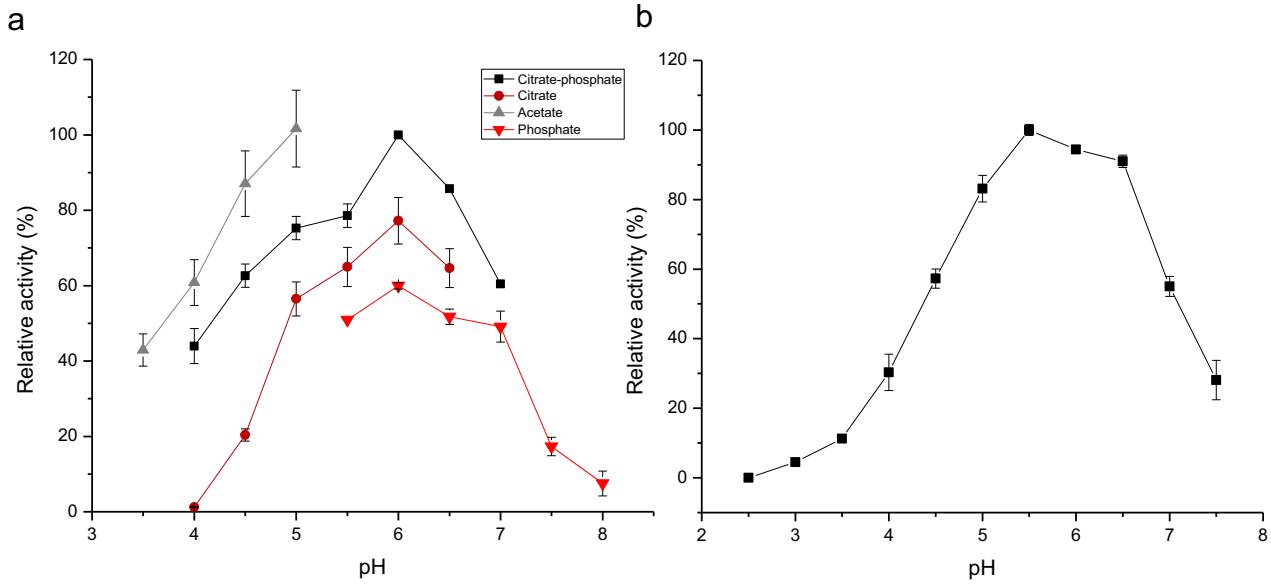


**Fig. 3.** (a) Effect of pH on the activity of TtBgnA using 2 mg/mL of laminarin in acetate buffer, citrate–phosphate buffer, citrate buffer, and phosphate buffer at various pH values, and temperature of 65°C. (b) pH stability of TtBgnA. Residual activity was determined at 70°C in citrate–phosphate buffer, pH 6.5, after 2.5 h of incubation at 25°C. Activity was estimated as a percentage of the maximum. Maximum activity was defined as 100%. The activity of the enzyme at 100% was 4.41 U/mg and 4.23 U/mg, for (a) and (b), respectively.

identity to an uncharacterized protein belonging to the GH31 family from *N. tetrasperma* FGSC 2508 (F8 N146), and 20.1% identity to α-glucosidase belonging to the GH31 family from *Chaetomium thermophilum* DSM 1495 (G0SG42).

The alignment of the GH31 protein from *T. terrestris* NRRL 8126 with the sequences of the top four matches from BLAST analysis is shown in Fig. S2. Alignment and comparison of the sequence of the GH31 protein from *T. terrestris* NRRLL 8126 with that of α-glucosidase belonging to the GH31 family from *C. thermophilum* DSM 1495 (G0SG42), which has been purified and crystallized (PDB 5DKX), have made possible the identification of 16 α-helices, 53 β-sheets, and the catalytic amino acid residues D700 and D868 characteristic of the GH31 family, identified by similarity (Fig. S2).

β-Glucanase from *T. terrestris* NRRL 8126 (UniProtKB: G2RFM7) was grouped with other β-glucanases from *M. thermophila* (G2Q431) and *Madurella mycetomatis* (A0A175VX48) of the GH31 family. The tree with the highest log likelihood (-5500.85) is shown in Fig. 8. All exo-β-1,3-glucanases of GH3, GH5, GH17, and GH55 families had strong nodal support values.

## 4. Discussion

β-1,3-Glucanases are enzymes mainly produced by organisms of the Fungi kingdom, and the majority of the fungal β-1,3-glucanases described are extracellular and hence secreted into the medium on synthesis [42]. A number of hypotheses have been proposed to

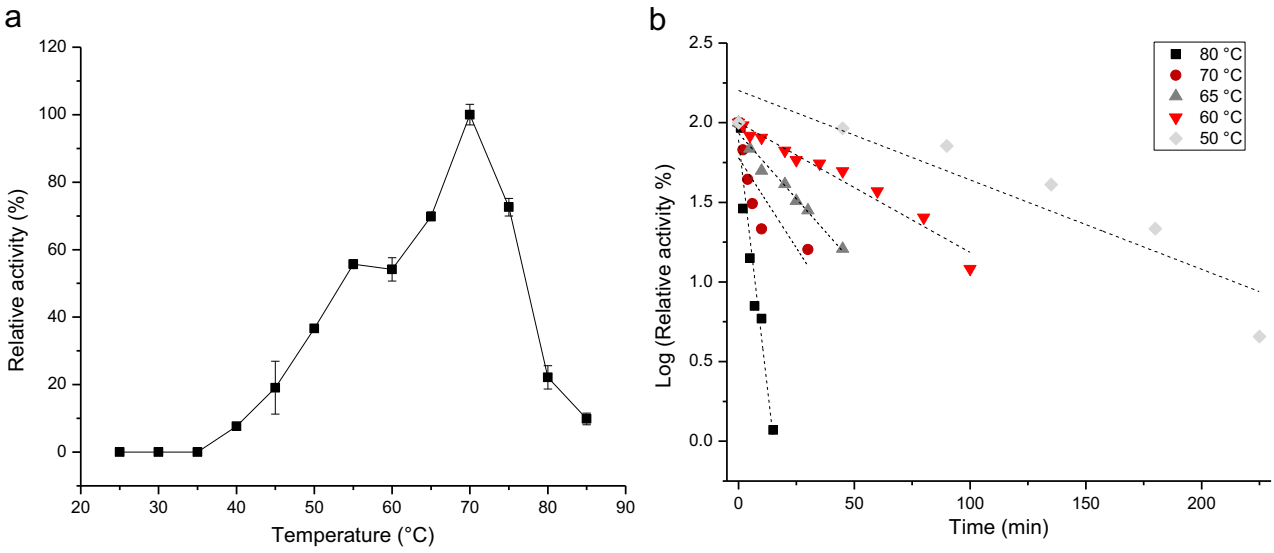


**Fig. 4.** (a) Effect of temperature on the activity of *Tt*BgnA using 2 mg/mL of laminarin in citrate–phosphate buffer, pH 6.5 at different temperatures ranging from 25 to 85°C. (b) Thermostability of *Tt*BgnA. Semi-log plot of residual activities (%) of *Tt*BgnA *versus* heating time for the determination of inactivation rate constant (*k*) values at 50, 60, 65, 70, and 80°C. Activity was estimated as a percentage of the maximum. Maximum activity was defined as 100%. The activity of the enzyme at 100% was 4.29 U/mg and 3.83 U/mg, for (a) and (b), respectively.

**Table 2**
Influence of metal ions on *Tt*BgnA activity.

| Metal ion | 1 mM[a] | 5 mM[a] |
|---|---|---|
| Control | 100 ± 7.1 | 100 ± 7.1 |
| $CaCl_2$ | 98 ± 0 | 95 ± 0 |
| CsCl | 111.84 ± 1.7 | 146.05 ± 8.9 |
| $CuCl_2$ | 103 ± 0 | 107 ± 0 |
| $FeCl_3$ | 169.73 ± 5.5 | 134.21 ± 2.8 |
| KCl | 94.73 ± 3.9 | 52.63 ± 7.1 |
| LiCl | 134.21 ± 8.3 | 203.94 ± 13.7 |
| $MnCl_2$ | 167.10 ± 7.8 | 0 |
| NaCl | 95 ± 0 | 91 ± 0 |
| $NiCl_2$ | 101.31 ± 5.5 | 136.84 ± 2.7 |

[a] Enzymatic activity was expressed as a percentage of the activity observed in the absence of any metal ion (control), which was taken as 100%. The activity of the enzyme at 100% was 4.35 U/mg.

explain how the fungus protects itself against its own lytic enzymes, *e.g.*, the existence of specific cell wall components such as oligosaccharides, which are able to act as inducers of certain lytic enzymes; there is even the possibility that the fungus prey may be weakened by a variety of secondary metabolites, in combination with hydrolytic enzymes. This secreted mixture may damage fungal hyphae, enabling the enzymatic release of oligosaccharides, which in turn would induce the expression of even more hydrolytic enzymes [43]. Finally, the idea of effector proteins that are involved in regulating the degradation and protection of the fungal cell wall itself has also been proposed [43]. Unfortunately, there is little information about the detailed cell wall composition of different fungi, which could help elucidate this process. On the other hand, it has been reported that the β-1,3-glucanases are of high importance for the degradation of the cell wall by phytopathogenic fungi in terms of various activities such as obtaining a carbon source [44]. Additionally, it has been stated that mycoparasitism is usually carried out by producing *haustoria*, which penetrate and absorb the hyphae nutrients from other fungi, *e.g.*, *Trichoderma* spp. and which are able to attack several fungi including some species that are related to *Trichoderma* [18].

β-1,3-Glucanases are of great importance because of their potential application in numerous industries and in the biological control of fungal pathogens in crops. We thus focused on the identification of at least one of the enzymes involved in the β-1,3-glucanase activity from the thermophilic biomass-degrading ascomycete *T. terrestris* Co3Bag1. First, we assessed the β-1,3-glucanase activity produced by this fungus in the presence of 1% glucose, 3% glucose, or 4% wheat bran as the carbon source; we found that the enzymatic activity was similar in the presence of 1% glucose and 4% wheat bran and the enzymatic activity was 5-fold lower with 3% glucose than with 1% glucose. Results with 3% glucose as the carbon source suggest the existence of carbon catabolic repression (CCR) for the production of β-glucanase activity by *T. terrestris* Co3Bag1. CCR is a global regulatory mechanism that

**Table 3**
Relative activities of *Tt*BgnA on different substrates.

| Substrate | Linkage | Relative activity (%)[a] |
|---|---|---|
| Laminarin | β-1,3/β-1,6 | 100 ± 7.2 |
| Gentiobiose | β-1,6 | 3.57 ± 0.7 |
| Curdlan | β-1,3 | 0 |
| Cellobiose | β-1,4 | 0 |
| Starch | α-1,4/α-1,6 | 0 |
| Glycogen | α-1,4/α-1,6 | 0 |
| Amylose | α-1,4 | 7.64 ± 0.5 |
| Maltose | α-1,4 | 0 |
| Saccharose | α-1,2 | 7.34 ± 0.4 |
| Methyl-α-D-glucopyranoside | α-link | 0 |
| Methyl-α-D-mannopyranoside | α-link | 4.00 ± 0.5 |

[a] Activity was estimated as a percentage of the enzymatic activity obtained with laminarin as the substrate, which was taken as 100%. The activity of the enzyme at 100% was 4.05 U/mg.

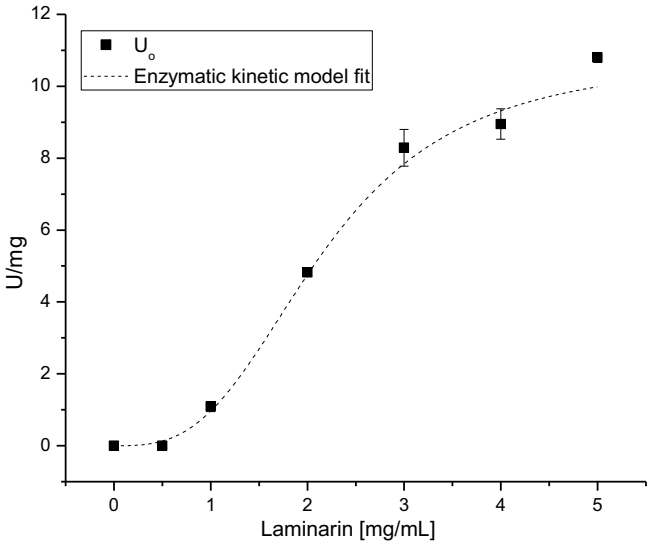


**Fig. 5.** Effect of substrate concentration on the activity of *Tt*BgnA. Kinetic parameters $K_M$ and $V_{max}$ were determined under optimum temperature and pH conditions. Laminarin concentration was 0.5 to 12 mg/mL.

occurs in microorganisms, but in the case of filamentous fungi, little is known, although some progress has been made in the study of the mediators of CCR in the devastating rice blast fungus *Magnaporthe oryzae* [45]. As β-1,3-glucanase activity appears to have been similar when *T. terrestris* Co3Bag1 was grown on 1% glucose and on 4% wheat bran, in this study, we decided to use 1% glucose as the carbon source for enzyme production, as it does not interfere with protein assay and enzyme purification.

Results from this work indicate that exo-β-1,3-glucanase activity from *T. terrestris* Co3Bag1 was evident throughout fermentation with 1% glucose, 3% glucose, and 4% wheat bran as carbon sources; however, in the case of 1% glucose, the highest exo-β-1,3-glucanase activity was displayed when glucose reached a low level. These findings concur with those reported in other studies that have grown fungi on glucose to produce exo-β-1,3-glucanase activity from *Acremonium* sp. [26,27]. For this fungi, the enzyme activity was registered throughout fermentation and the highest exo-β-1,3-glucanase activity was observed when the carbon source was at a low level, and the extracellular exo-β-1,3-glucanase activity was also related to mycelial growth [46,47].

*Tt*BgnA, a β-1,3-glucanase with an estimated molecular mass of 129 kDa, was purified from the culture supernatant of the filamentous fungus *T. terrestris* Co3Bag1, grown on 1% glucose as the carbon source. The enzyme was purified by two steps of conventional chromatographic methods, AEC and GFC. The enzyme yield was too low, probably due to the presence of proteolytic activity in the culture supernatant of this fungus, used as a source of β-1,3-glucanase activity. Thus, the use of *Tt*BgnA in industrial processes requires a better purification process strategy that results in a reasonable level of enzyme yield, although the use of a concentrated preparation of fungal enzymes by ultrafiltration and their use for industrial processes has been reported [48].

Purified *Tt*BgnA exhibited a specific activity of 6.0 U/mg, which was 22-fold greater than that reported for the exo-β-1,3-glucanases from *Rhizoctonia solani* (0.265U/mg) [15] and similar to the specific activity of the exo-β-1,3-glucanase from *T. asperellum* (7.7 U/mg) [49].

The β-1,3-glucanase *Tt*BgnA from *T. terrestris* Co3Bag1 was biochemically characterized. *Tt*BgnA displayed optimal activity at pH 6.0, which is similar to that reported for other fungal glucanases, *e.g.*, exo-β-1,3-glucanases from *F. imperfecti* [50], *Achlya bisexualis* [51], *Chaetomium* sp. [52], *Pichia pastoris* [53], *Periconia byssoides* [54], and *C. thermophilum* [34]. Likewise, the enzyme displayed more than 50% of its maximal activity at pH values ranging from 4 to 7. pH stability assays

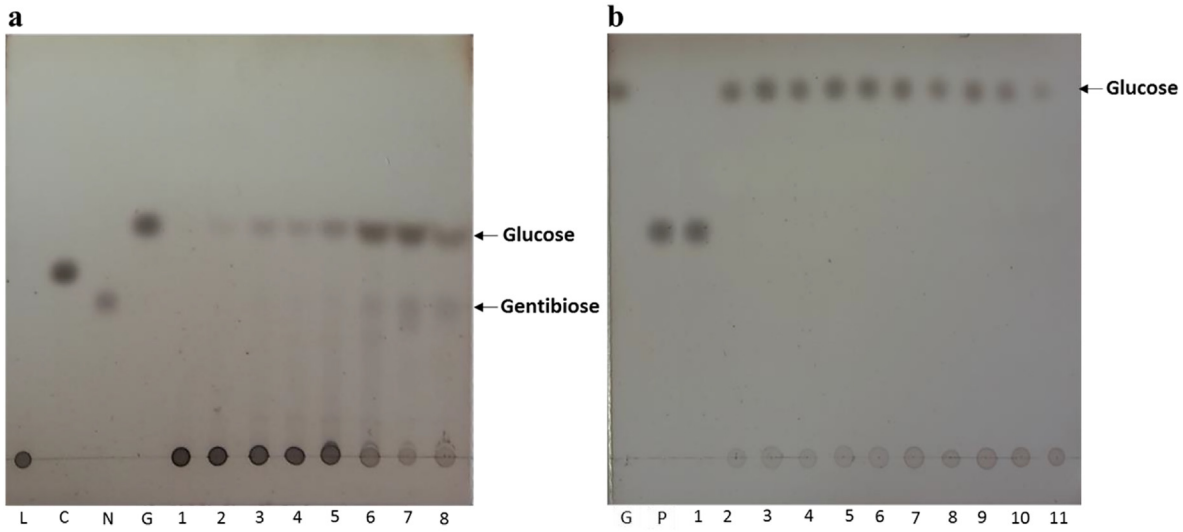


**Fig. 6.** TLC analysis of the products of hydrolysis of laminarin and *p*NPG by *Tt*BgnA in citrate–phosphate buffer, pH 6.0 at 40°C. (a) Standards: laminarin (L), cellobiose (C), gentiobiose (N), and glucose (G); lanes (1–8) show different reaction times for hydrolysis (0, 0.2, 0.4, 0.6, 2, 6, 12, and 48 h, respectively). (b) Standards: glucose (G), *p*NPG (P); lanes (1–11) show different reaction times for hydrolysis (0, 0.2, 0.4, 0.6, 2, 4, 6, 8, 12, 24, 48 h, respectively).

indicated that *Tt*BgnA remained active within the of pH range of 4.5–7.0, after preincubation at 25°C for 2.5 h, whereas other fungal β-1,3-glucanases were usually stable for a few minutes, within the pH range of 4.0–7.0; for example, β-glucanase from *C. thermophilum* [34]. A decline in enzymatic activity at different pH values may be due to the change in the ionization state of certain amino acid residues, possible leading to conformational changes in the overall 3D structure of the enzyme, including the active site, thus affecting its catalytic activity [55].

*Tt*BgnA exhibited optimal activity at 70°C; hence, it may be considered as a thermophilic enzyme; additionally, *Tt*BgnA possibly represents the fungal β-1,3-glucanases that displays the higher optimal temperature reported thus far (Table 5). The optimal temperature of *Tt*BgnA is higher than those reported for exo-β-1,3-glucanases from *Acremonium persicinum* (65°C) [56], *Rasamsonia emersonii* (65°C) [3], *Aspergillus phoenicis* (50°C) [57], and *Chaetomium* sp. (30°C) [52]. The thermal stability of *Tt*BgnA ($t_{1/2}$ of 54 and 37 min at 50 and 60°C, respectively) was greater than that previously reported for the exo-β-1,3-glucanase from *Saccharomyces cerevisiae*, which was stable at 50°C for 30 min, but after this time, enzyme activity ceased [58], whereas exo-β-1,3-glucanase from *T. reesei* lost all its activity when incubating at 60°C for 15 min [59], and the exo-β-1,3-glucanase from *Chaetomium* sp. became inactive above 60°C (for 30 min) [52]. Nevertheless, the thermal stability of *Tt*BgnA at higher temperatures was lower than that reported for the exo-β-1,3-glucanase from *C. thermophilum* ($t_{1/2}$ of 55 and 21.5 min at 65 and 70°C, respectively) [34] but similar to that observed for the exo-β-1,3-glucanase from *Chaetomium* sp. ($t_{1/2}$ of 5 min at 80°C) [52].

Interestingly, the β-1,3-glucanase activity of *Tt*BgnA was increased to 134%, 167%, and 169% in the presence of $Li^{1+}$, $Mn^{2+}$, and $Fe^{3+}$ (1 mM each), respectively, whereas, an increase of 200% was registered with 5 mM $Li^{1+}$. Likewise, an increase to 181% in the activity of fungal exo-β-1,3-glucanase from *Cyberlindnera saturnus* was reported in the presence of 10 mM $Li^{1+}$ [60]. In agreement with findings here, an increase to 186% in the activity of an exo-β-1,3-glucanase from *T. viride* was registered in the presence of 1 mM $Mn^{2+}$ [61]. Thus far, there is no clear pattern to predict which ion will lead to a dramatic improvement or reduction in enzyme activity [62].

Purified *Tt*BgnA was able to hydrolyze laminarin and, to a lesser extent, gentiobiose (β-1,6-glucan link), amylose (α-1,4 link), and saccharose (α-1,2 link) (3–7% relative to laminarin) under standard assay conditions (at 70°C, pH 6.0); however, the enzyme was not active on cellobiose (β-1,4 link), starch, and glycogen (α-1,4/α-1,6 links). Furthermore, TLC analysis of hydrolysis products released from saccharose suggested the enzyme was not able to hydrolyze saccharose under the experimental conditions of the assay (at 40°C, pH 6.0) (Fig. S1). This means that the low level of activity of *Tt*BgnA with gentiobiose (β-1,6-glucan link), amylose (α-1,4 link), and saccharose (α-1,2 link) as the substrates may be partly due to a lack of stereoselectivity displayed by the enzyme under the conditions tested in this work. In fact, it has been found that enzymes are not completely stereoselective, especially in the case of those that hydrolyze polysaccharides. In agreement with our data, it has been reported that some β-1,3-glucanases have low activity on substrates with other types of link, *e.g.*, Pustulan [3], cellobiose, cellotriose, gentiobiose, lichenan, pustulan [4], and curdlan, lichenan, and pullulan [63]. Thus, findings here suggest that *Tt*BgnA has high affinity with the β-1,3-glucan links present in laminarin (β-1,3/β-1,6 link). These findings concur with those previously reported for β-1,3-glucanases, *e.g.*, from *P. chrysosporium* [64], *T. emersonii* [3], *Penicillium* sp. [65], *A. persicinum* [55], *S. cerevisiae* [58], and *P. pastoris* [53]. *Tt*BgnA exhibited a low level of activity on gentiobiose; likewise, a low level of activity was found for an exo-β-1,3-glucanase from *P.*

**Table 4**
Blast search of peptide sequences from purified *Tt*BgnA.

| number | Peptide[a] | % identity |
|---|---|---|
| 1 | GRFPGTGPFPSRDTNTNTSASSAELTSYPAWPPR | 85.3 |
| 2 | LWFTGFCLRAASGDYDHSSVSR | 90.9 |
| 3 | NVLPNIIDPKAVDPQTVCPGYK | 95.5 |
| 4 | QADDRVHVDIRPRFISPENESWFLLPEALVPRP | 97 |
| 5 | QSDSALIVSWSNDPTFSFSVK | 100 |
| 6 | VLVYEDQFIEFASALPERYNLYGLGEVIHGFR | 96.9 |
| 7 | TLFAADVGDTIDANLYGSHPIYLDTR | 100 |
| 8 | YLSYTHGVFLRNAHAQEILLRPSSVTWRTLGGSIDLYFYSGPK | 97.7 |
| 9 | AYQQTAVGLPAMQQYWSLGFHQCR | 100 |
| 10 | SWAEVEDVVDNFAKFEIPLETIWTDIDYMKQYRDFENDPVR | 97.6 |
| 11 | FLAKLHANHQHYVPIIDSAIYAPNPENPDDAYPPYDR | 100 |
| 12 | GGYLEYDVHNLFGH | 100 |
| 13 | QALLKVHEGKRPFIIGR | 100 |
| 14 | WMQLSAFFPFYRNHNILGAISQEPYVWSSVAEATK | 100 |
| 15 | YSLLPYMYTLMARASLEGSTVMRALAWEFPAEPWLADADR | 100 |
| 16 | GGSVVPTQEPGMTTTESRRNPWGLIVALDR | 96.7 |
| 17 | SATNNSVVARPEGNYVDTNR | 95 |
| 18 | AEAPSKVWLNGDLLATSRWSYSADRA | 88 |

[a] All the peptides generated from the sequencing are shown to have identity with the glycoside hydrolase family 31 protein of *Thielavia terrestris* NRRL 8126 and UniProtKB accession number G2RFM7.

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MEPAASPTRAARPLAVKHKGRFPGSRPFPSRDTNTYTSTSSAEITSYPAWPPRRSVVGIR    60
                    GRFPGTGPFPSRDTNTNTSASSAELTSYPAWPPR
GCTGLVQSAAPKSKSPPPLWRSSSTATLVQSVLILVGGIATAFAEGTGILDVTLDAEEQD   120
VQLQEGPDAYIGEPTKLDQVGRAPQHSGTWILGLGIYIPLGLFLLLFLFALTYSFKRRSR   180
IPRSKATGSSRFIQSAIAMFSFTLLLWITGFCLRAASGDYDHSAVSRTSWQPNQTVAPAP   240
                          LWFTGFCLRAASGDYDHSSVSR
VFTIPASATLGRNVLPNIIDPEAVDPQTVCPGYKASNIDETEGGFTAELDLAGPACNVYG   300
                 NVLPNIIDPKAVDPQTVCPGYK
NDIEHLSLSVDFQADDRVHVDIRPRFISPENESWFLLPEVLVPRPPRGARYQQSDSALIV   360
                 QADDRVHVDIRPRFISPENESWFLLPEALVPRP      QSDSALIV
SWSNDPTFSFSVKRRETNDTLFSTEGKVLVYEDQFVEFASALPERYNLYGLGEVIHGFRL   420
SWSNDPTFSFSVK              VLVYEDQFIEFASALPERYNLYGLGEVIHGFR
GDNLTRTLFAADVGDTIDANLYGSHPIYLDTRYFVADDSGELTYVQNTTDKANKYVSYTH   480
      TLFAADVGDTIDANLYGSHPIYLDTR                        YLSYTH
GVFLRNAHAQEILLRPSSVTWRTLGGSIDLYFYSGPKAQDVIRAYQQTAVGLPAMQQYWS   540
GVFLRNAHAQEILLRPSSVTWRTLGGSIDLYFYSGPK     AYQQTAVGLPAMQQYWS
LGFHQCRWGYKSWAEVEDVVDNFARFEIPLETIWTDIDYMKQYRDFENDPVRFGYTEGSK   600
LGFHQCR    SWAEVEDVVDNFAKFEIPLETIWTDIDYMKQYRDFENDPVR
FLAKLHANHQHYVPIIDSAIYAPNPENPDDAYPPYDRGVEAKAFMLNPDGSIYYGAVWPG   760
FLAKLHANHQHYVPIIDSAIYAPNPENPDDAYPPYDR
YTVFPDWVGAVLDGGGAIDWWIDEILRYSKKVAFDGIWIDMSEVSSFCVGSCGTGNLTLN   720
PAHPPFELPGEPGNLVLQYPEGFNSTNSSEAMSASSASRQQEAATATTQAPTSTSYYRTT   780
PTSGARNVNWPPYVINNSNGELAGHAVSPNATHHGGYLEYDVHNLFGHMILNATYQALLK   840
                                GGYLEYDVHNLFGH      QALLK
VHEGKRPFIIGRSTFAGSGKWAGHWGGDNAALWAYMYFSIPQALSFSIFGIPMFGVDTCG   900
VHEGKRPFIIGR
FAGNTDYELCSRWMQLSAFFPFYRNHNILGAISQEPYVWSSVAEATKTAMRIRYSLLPYM   960
          WMQLSAFFPFYRNHNILGAISQEPYVWSSVAEATK      YSLLPYM
YTLMARASLEGSTVMRALAWEFPAEPWLADADRQFMLGSALMVTPCLEQGASTVGVVFPG  1020
YTLMARASLEGSTVMRALAWEFPAEPWLADADR
VGDGTVWYDWYTLAAVKGVEPGQNVTIDAPLGHIPLYLRGGNVVPTQEPGMTTTESRRNP  1080
                                GGSVVPTQEPGMTTTESRRNP
WGLIVALDRDGSAEGELYLDDGESLEPDAVTWVHFSATNNSVAARPEGNYVDTNRLANVT  1140
WGLIVALDR                       SATNNSVVARPEGNYVDTNR
VLGLPEAPSMVWLNGNLLATSRWSYSADSAVLSVQGLHDLSPEGAWAAAWELTWE       1195
    AEAPSKVWLNGDLLATSRWSYSADRA
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**Fig. 7.** Alignment between the amino acid sequence of glycoside hydrolase family 31 from *T. terrestris* NRRL 8126 (UniProtKB accession number G2RFM7) (continuous sequence) and the 18 peptides generated from the sequencing of *Tt*BgnA by MS/MS (underlined and boldfaced). Conserved amino acid residues are highlighted with gray boxes.

*pastoris* with gentiobiose as the substrate [53]. Interestingly, *Tt*BgnA did not manifest activity on curdlan, even when this substrate also had β-1,3-links. In agreement with this finding, it has been reported that some β-1,3-glucanases manifest intense activity on laminarin but little or no activity on other β-1,3-linked polysaccharides, *e.g.,* for β-1,3-glucanases from *A. persicinum* [56], *Ganoderma tsugae* [66], *Hordeum vulgare* [4], *A.*

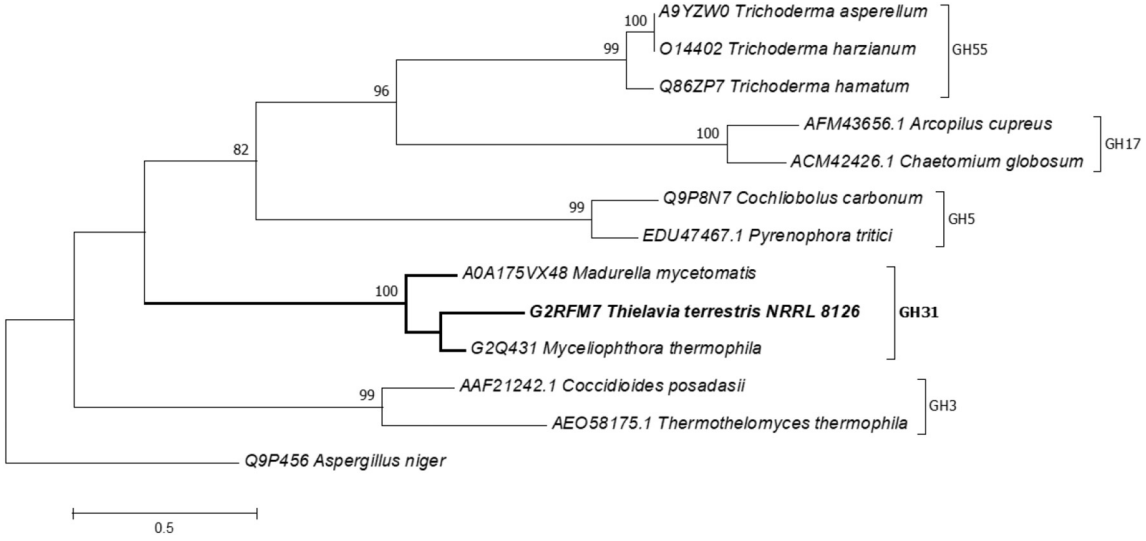


**Fig. 8.** Maximum-likelihood tree of β-glucanase from *Thielavia terrestris* NRRL 8126 with other β-glucanases of GH31 family and exo-β-1,3-glucanases of GH3, GH5, GH17, and GH55 families. The analysis involved 13 amino acid sequences, and accession numbers of UniProtKB and NCBI database are shown in the branches of the tree.

**Table 5**
Comparison of some of the biochemical properties of fungal β-1,3-glucanases and *Tt*Bgn31A.

| Microorganism | Protein name | Optimal Temperature (°C) | Thermal stability ($t_{1/2}$) | Optimal pH | pH stability | Substrate | Hydrolysis products (TLC) | Kinetic parameters | Ref. |
|---|---|---|---|---|---|---|---|---|---|
| *T. terrestris Co3Bag1* | *Tt*Bgn31A | 70 | 50 °C, 54 min<br>60°C, 37 min<br>65 °C, 18 min<br>70°C, 13 min<br>80°C, 2 min | 6.0 | 4.5–7.0 | Laminarin | Laminarin (glucose) | $k_M$ (2.2 mg/mL)<br>$V_{max}$(10.8 U/mg) | This work |
| *Chaetomium* sp. | β-1,3-glucanase | 30 | 80°C, 5 min<br>60°C (inactive over 30 min) | 6.0 | 5.0–7.0 | Laminarin | ND | ND | [52] |
| *P. byssoides* | glucan 1, 3-β-glucosidase | 50 | 30 °C, 40% of maximal activity<br>80°C, 65% of maximal activity | 6.0 | 5.0–7.0 | Laminarin | ND | ND | [54] |
| *C. thermophilum* | Glucan 1,3-β-glucosidase | 60 | 65 °C, 55 min<br>70°C, 21.5 min<br>80°C, 5 min | 6.0 | 4.0–7.0 | Laminarin | ND | ND | [34] |
| *A. persicinum* | GN1 | 65 | ND | 5.0 | 5.0–7.5 | Laminarin | Laminarin (glucose, laminaribiose and laminaritriose) | $k_M$ (0.1 mg/mL)<br>$V_{max}$ (261 U/mg) | [56] |
| *R. emersonii* | exo-1,3-β-glucanase | 65 | ND | 5.4 | 2.8–8.0 | Laminarin | Laminarin (glucose) | $k_M$ (1.66 mg/mL)<br>$V_{max}$ (7.69 U/mL) | [3] |
| *A. phoenicis* | exo-1,3-β-glucanase | 50 | ND | 3.8 | ND | Laminarin | Pachyman (glucose) | $k_M$ (2.5 mg mL) | [57] |
| *T. reesei* | Laminarinase | ND | 60 °C, 15 min | 5.5 | ND | Laminarin | ND | $k_M$ (0.28 mg/mL) | [59] |
| *S. cerevisiae* | Exg 1p | 40 | 50 °C, 30 min | 5.8 | 3.0–10.0 | Laminarin | ND | $k_M$ (13 mg/mL)<br>$V_{max}$ (1.24 μM/s) | [58] |
| *T. asperellum* | Tag83 | 40 | ND | 5 | ND | Laminarin | Laminarin (glucose) | ND | [49] |
| *R. solani* | Exo-1, 3-β-glucanase | 40 | ND | 5.0 | 3.0–7.0 | Laminarin | ND | $k_M$ (0.78 mg/mL)<br>$V_{max}$ (0.27 U/mg) | [26] |

ND: Not Determined.

*fumigatus* [67], *P. chrysosporium* [64], *Acremonium blochii* [68], *Penicillium* sp. [65], *Podospora anserina* [6], and *Penicillium rolfsii* [63].

Apparent $K_M$ and $V_{max}$ values for purified *Tt*BgnA were determined using laminarin as the substrate. The $K_M$ value of *Tt*BgnA for laminarin (2.2 mg $mL^{-1}$) is similar to the $K_M$ value reported for the exo-β-1,3-glucanase (2.5 mg $mL^{-1}$) from *A. phoenicis* [57], and lower than that previously reported for fungal exo-β-1,3-glucanases from *Schizosaccharomyces japonicus* (6.25 mg $mL^{-1}$) [69], and *Candida albicans* (3.8 mg $mL^{-1}$) [70]. The $V_{max}$ value of *Tt*BgnA (10.8 U/mg) was higher than the $V_{max}$ value from *R. solani* (0.27 U/mg) [15] and lower than that from *P. anomala* (350 U/mg) [71].

Based on TLC analysis, *Tt*BgnA represents an exo-type enzyme defined by its ability to release mainly glucose and gentiobiose from laminarin and glucose from *p*NPG. It is worth mentioning that according to Fig. 6b, the longer hydrolysis time of *Tt*BgnA on *p*NPG results in a lower glucose content. This is an unexpected but reproducible result that may be due to a chemical modification of glucose after 8 to 48 h of incubation at 40°C, which apparently does not occur during the first 8 h of incubation at the same temperature; thus, for a better interpretation of these results, a quantitative analysis of the hydrolysis products released from pNPG by the action of *Tt*BgnA by HPLC or gas chromatography may be useful to confirm the presence of glucose and glucose-derived compounds. Several fungal β-1,3-glucanases with exo-activity on laminarin and *p*NPG have been previously reported, *e.g.,* the exo-β-1,3-glucanases from *T. emersonii* [3], *T. asperellum* [49], and *C. albicans* [72]. Experiments have revealed that purified exo-β-1,3-glucanases may hydrolyze both laminarin and *p*NPG, but the endo-β-1,3-glucanases did not hydrolyze *p*NPG [73]. In addition to this, endo-β-1,3-glucanases produce laminarian oligosaccharides from laminarins, *e.g.*, the endo-β-1,3-glucanases from *A. fumigatus* [67].

The GH31 protein from *T. terrestris* NRRL 8126 showed conserved residues that are characteristic of the GH31 family (Fig. S1), thus suggesting that this enzyme can be classified as a possible member of the GH31 family. Additionally, the GH31 protein from *T. terrestris* NRRL 8126 shows closer relationship with β-1,3-glucanases of GH5, GH17, and GH55 families than with those of the GH3 family, as shown by the phylogenetic tree (Fig. 8). The GH5 and GH55 families have a greater evolutionary relationship with glucanases that manifest exo-β-1,3 hydrolytic activity [7]. Recently, the identification and phylogenetic inclusion of β-1,3-glucanases in other GH families have been reported [6]. This implies that *Tt*BgnA from *T. terrestris* Co3Bag1 can be classified as a member of the family GH31 of the glycosyl hydrolase superfamily; this is the first enzyme pertaining to the GH31 family to manifest β-1,3 hydrolytic activity.

This report describes the purification and characterization of *Tt*BgnA from *T. terrestris* Co3Bag1, possibly constituting the first exo-glucanase from the GH31 family to manifest β-1,3 hydrolytic activity. A comparison of certain biochemical properties of fungal β-1,3-glucanases, including those exhibited by the β-1,3-glucanase *Tt*BgnA (this work), is shown in Table 5. *Tt*BgnA appears to be a good candidate for use in industrial processes, as it has an optimum temperature at 70°C, which complies with most industrial processes that require enzymes that tolerate temperatures ranging from 55 to 70°C.

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## Conflict of interest

The authors declare that they have no conflict of interest.

## Supplementary material

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