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Biochemical characterization of a 27 kDa 1,3-β-D-glucanase from *Trichoderma asperellum* induced by cell wall of *Rhizoctonia solani*

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ABSTRACT

Trichoderma asperellum produces two extracellular 1,3-β-D-glucanase upon induction with cell walls from *Rhizoctonia solani*. A minor 1,3-β-D-glucanase was purified to homogeneity by ion exchange chromatography on Q-Sepharose and gel filtration on Sephacryl S-100. A typical procedure provided 13.8-fold purification with 70% yield. SDS–PAGE of the purified enzyme showed a single protein band of molecular weight 27 kDa. The enzyme exhibited optimum catalytic activity at pH 3.6 and 45 °C. It was thermostable at 40 °C, and retained 75% activity after 60 min at 45 °C. The K_m and V_{max} values for 1,3-β-D-glucanase, using laminarin as substrate, were 0.323 mg ml^{-1} and 0.315 U min^{-1}, respectively. The enzyme was strongly inhibited by Hg^{2+} and SDS. The enzyme was only active toward glucans containing β-1,3-linkages. Peptide sequences showed similarity with two endo-1,3(4)-β-D-glucanases from *Aspergillus fumigatus* Af293when compared against GenBank non-redundant database.

1. Introduction

Trichoderma fungi are well known for their antagonism against several soil–phytopathogens fungi. Their biological control activity is mainly attributable to various antimicrobial compounds and hydrolytic enzymes, in addition to their aggressive mode of growth and physiology (Harman, Howell, Viterbo, Chet, & Lorito, 2004; Schuster & Schmoll, 2010). The majority of *Trichoderma* preparations used commercially for biological control are from *T. atroviride* or *T. harzianum*. However, *T. asperellum*, a less well-studied species, is also an effective biological control agent against rice seed-borne diseases (Watanabe, Kato, Kumakura, Ishibashi, & Nagayama, 2006), *Rhizoctania solani* (Trillas et al., 2006), *Fusarium* spp. (Sant et al., 2010). The interaction of *T. asperellum* with *R. solani* is characteristically mycoparasitic, involving growth along the host hyphae and coiling (Harman et al., 2004).

Most phytopathogenic fungi have cell wall that contain chitin as a structural backbone arranged in regularly ordered layers and β-1,3-glucan as a filling material arranged in an amorphous manner (Adams, 2004). Chitinases and 1,3-β-D-glucanase have been found to be directly involved in the mycoparasitism interaction between *Trichoderma* species and its hosts (Harman et al., 2004). Several types of β-glucan-degrading enzymes exist, classified according to the type of β-glucosidic linkage they cleave and mechanism of substrate attack (Martin, McDougall, McIlroy, Chen, & Seviour, 2007). They can hydrolyze the substrate by two possible mechanisms, identified by the products of hydrolysis: (a) exo-β-glucanases hydrolyze the substrate by sequentially cleaving glucose residues from the non-reducing end, and (b) endo-β-glucanase cleave β-linkages at random sites along the polysaccharide chain, releasing smaller oligosaccharides.

T. asperellum produces two extracellular 1,3-β-D-glucanase upon induction with cell walls from *R. solani* (Bara, Lima, & Ulhoa, 2003). However, little is known about the biochemistry of 1,3-β-D-glucanase produced by *T. asperellum*. We have previously described the purification and characterization of an exo-1,3-β-D-glucanase with apparent molecular weight of 83.1 kDa (Bara et al., 2003). Recently, we showed that the expression of exo-1,3-β-D-glucanase gene (*tag83*) is significantly increased during *in vivo* assay against *R. solani* (Marcello et al., 2010). In this study, we report the purification, characterization and identification of a minor extracellular 1,3-β-D-glucanase produced by *T. asperellum* after growth in presence of cell wall of *R. solani*.

2. Materials and methods

2.1. Organism and culture conditions

T. asperellum (T00) was isolated from Cerrado soil of the central region of Brazil (Enzymology Group collection, UFG/ICB). Its

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identification was made by amplification of nuclear rRNA region containing the ITS1 and ITS2 and the 5.8S rRNA gene using the primer combination SR6R and LR1, following the protocol described by White, Bruns, Lee, & Taylor (1990). Sequence analysis of the ITS amplicon was performed using the TRICHOKEY 2.0 (Druzhinina & Kopchinskiy, 2006; Druzhinina et al., 2005). Spores from *T. asperellum*, were collected in sterile saline, centrifuged at 2000 × *g*, washed twice and used as inoculum (1.0×10^7 spores ml^{-1} in liquid medium-TLE). TLE medium contained: 0.1% (w/v) bactopeptone, 0.03% (w/v) urea, 0.2% (w/v) KH_2PO_4, 1.4% (w/v) $(NH_4)_2SO_4$, 0.03% (w/v) $MgSO_4{\cdot}7H_2O$ and trace elements solution containing Fe^{2+}, Zn^{2+}, Mn^{2+}, Cu^{2+}. Glucose 1% (w/v), glucose 2% (w/v), 0.5% (w/v) starch, 0.5% (w/v) cellulose, 0.5% (w/v) chitosan, 0.5% (w/v) chitin and 0.5% (w/v) cell walls of *R. solani* were used as carbon source. The cultures were grown in conical flasks with constant shaking (180 rpm) at 28 °C for 24 h. The mycelium was harvested by filtration through filter paper, and the culture filtrate was dialyzed overnight against distilled water, freeze-dried and used as source of 1,3-β-D-glucanases. Purification of cell wall from *R. solani* was made by the method described by Mitchell and Taylor (1969).

2.2. 1,3-β-D-glucanase assay

Enzyme activity was measured by mixing 50 μl of sample with 100 μl of 50 mM acetate buffer (pH 5.0), containing 0.25% (w/v) laminarin (Sigma). The mixture was incubated at 40 °C for 30 min and the reducing sugar produced was determined by the method described by Miller (1959). One unit (U) of 1,3-β-D-glucanase activity was defined as the amount of enzyme that produced 1 μM of reducing sugar min^{-1} under the above conditions. Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as standard.

2.3. Enzyme purification

The crude enzyme was concentrated by ultrafiltration using a 10 kDa membrane. The concentrated samples were loaded on Q-Sepharose Fast Flow column (1.5 × 18.4 cm) equilibrated with sodium acetate buffer (50 mM, pH 5.0), and eluted at a flow rate of 60 ml h^{-1}. The column was washed with the same buffer and eluted with a linear gradient of 0–0.5 M NaCl. Fractions of 5.0 ml were collected and monitored for protein (A_{280}) and 1,3-β-D-glucanase activity. Fractions containing 1,3-β-D-glucanase activity were pooled and applied directly onto a Sephacryl S-100 column (2.5 × 48 cm) previously equilibrated with sodium acetate buffer (50 mM, pH 5.0), and eluted with the same buffer at a flow rate of 40 ml h^{-1}. Fractions containing 1,3-β-D-glucanase activity were pooled, dialyzed against water and stored at −20 °C.

2.4. Electrophoretic analysis and protein identification

Polyacrylamide gel electrophoresis (SDS–PAGE) was used to determine protein purity and the molecular mass of the purified enzyme under denaturing conditions using a 12% (v/v) acrylamide gel, as described by Laemmli (1970). Protein was silver stained as described by Blum, Beier, & Gross (1987). Molecular weight markers (Thermo Scientific) were as follow: β-galactosidase (116 kDa), Bovine serum albumin (66.2 kDa), Ovalbumin (45 kDa), Lactate dehydrogenase (35 kDa) and REase Bsp98I (25 kDa).

2.5. Activity detection of 1,3-β-D-glucanase after non-denaturing electrophoresis

Zymograms were carried out as described by Pan, Ye, & Kue (1989). After non-denaturing PAGE, gels were washed with distilled water, incubated with 50 mM sodium acetate (pH 5.0) for 60 min, and then incubated with at 40 °C for 180 min in a solution containing 0.75% (w/v) of laminarin (in 50 mM sodium acetate, pH 5.0). Protein bands with 1,3-β-D-glucanase activity were visualized after boiling the gel with 2,3,5-triphenyltetrazolium chloride solution (TTC).

2.6. Enzyme characterization

The effect of pH on the enzyme activity was determined by varying the pH of the reaction mixtures using 100 mM citrate/phosphate buffer (pH 2.0–7.0). The effect of temperature on the enzymatic activity was determined at pH 3.6, in the range 25 °C to 60 °C. The effects of $HgCl_2$ and SDS on 1,3-β-D-glucanase activity were determined after pre-incubation with the respective compound (2.5 mM) for 5 min at 45 °C. Kinetics parameters of the purified 1,3-β-D-glucanase were estimated for laminarin by using a concentration ranging from 50 to 300 μg ml^{-1}. Activities were determined by the standard procedure and kinetics parameters (K_m and V_{max}) were calculated from Linewever–Burk plots by using the ENZIFIX©program. The ability of *T. asperellum* 1,3-β-D-glucanase to hydrolyze different substrates, i.e. laminarin, CWRS, cellulose, chitin, chitosan and starch was examined at substrate concentrations of 0.5% (w/v).

2.7. In-gel digestion and mass spectrometry

The purified enzyme was visualized in a SDS–PAGE (12%) stained with PhastGel™ Blue R (GE Healthcare). The protein band was excised, cut in pieces and in-gel digestion performed for further mass spectrometric analysis. Briefly, 200 μl of 25 mM NH_4HCO_3/50% (v/v) acetonitrile was added to gel pieces, agitated for 20 min, and supernatant discarded. This step was performed twice. 100 μl of 100% acetonitrile was added, agitated for 5 min, discarded and gel pieces vaccum-dried. Then, 10 μl of 25 mM NH_4HCO_3/10 mM DTT was added to gel pieces, incubated at 56 °C for 1 h and solution discarded. 25 mM NH_4HCO_3/55 mM iodoacetamide was added, incubated for 45 min at room temperature and discarded. 100 μl of 25 mM NH_4HCO_3 was added to gel pieces, agitated for 10 min, replaced by 100 μl of 25 mM NH_4HCO_3/50% (v/v) acetonitrile, agitated for 5 min. Solution was discarded and gel pieces vaccum-dried. The gel pieces were swollen at 4 °C in 15 μl of 25 mM NH_4HCO_3 containing 10 μg μl^{-1} of trypsin (Trypsin Gold, mass spectrometry grade, Promega) for 15 min. 40 μl of 25 mM NH_4HCO_3 was added to gel pieces and incubated at 37 °C for 16 h. The generated peptides were extracted with 100 μl of 100% (v/v) acetonitrile/5% (v/v) trifluoroacetic acid (2 steps of 10 min each) and vacuum-dried.

The resulting peptides were submitted to mass spectrometric analysis, which was carried using an Ultraflex III MALDI-TOF/TOF mass spectrometer (Bruker Daltonics), controlled by the FlexControl 3.0 software (Bruker Daltonics).

Peptides obtained were mixed with matrix solution (10 mg ml^{-1} of α-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile/0.3% (v/v) trifluoroacetic acid), directly into a MTP AnchorChip 400/384 target plate and dried at room temperature. Protein average masses were obtained in linear mode with external calibration, using the Protein Calibration Standard (Bruker Daltonics). The peptide monoisotopic masses were obtained in reflector mode with external calibration, using the Peptide Calibration Standard (Bruker Daltonics). Peptide MS/MS spectra were obtained by means of LIFT fragmentation. The software PepSeq (Waters, Manchester, UK) was used for mass spectrometric data analysis. Peptide primary structures were inferred by means of manual *de novo* interpretation of fragmentation spectra. The search for similar sequences was performed with the Protein Blast tool (blastp algorithm), using the

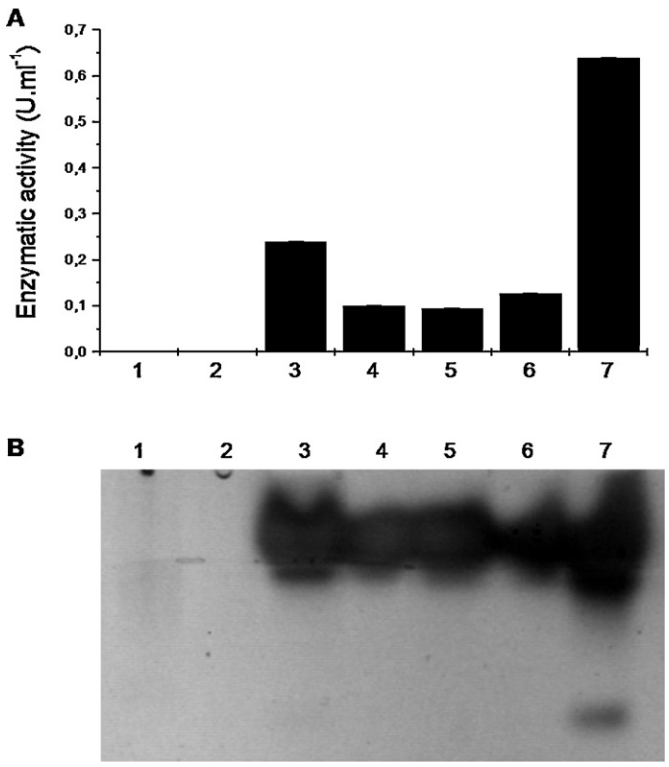

Fig. 1. Analysis of 1,3-β-glucanase activity when *T. asperellum* was grown on glucose 1% (1), glucose 2% (2), starch (3), cellulose (4), chitosan (5), chitin (6) and cell walls of *R. solani* (6). (A) Enzyme activity (U ml^{-1}); (B) detection of extracellular exo-1,3-β-D-glucanase activity on non-denaturing PAGE.

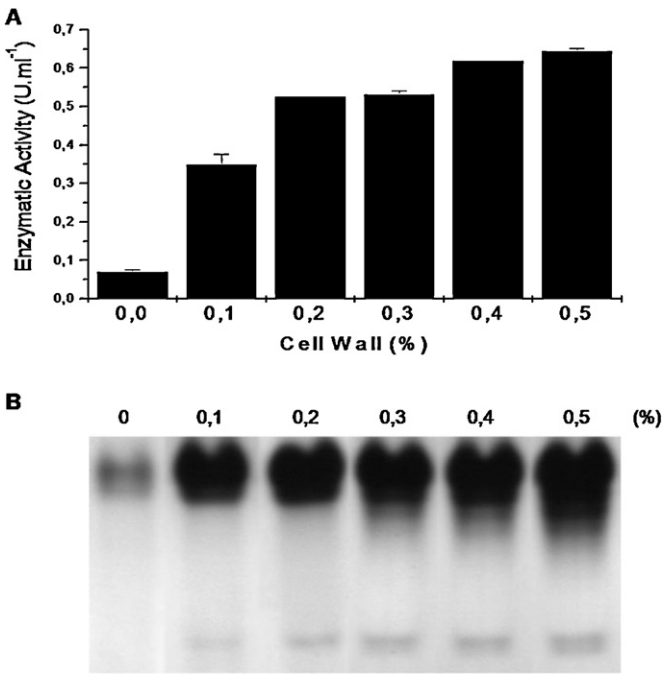

Fig. 2. Analysis of 1,3-β-glucanase activity when *T. asperellum* was grown on different concentrations of cell walls of *R. solani*. (A) Enzyme activity (U ml^{-1}); (B) detection of extracellular 1,3-β-glucanase activity on non-denaturing PAGE.

GenBank non-redundant database (www.ncbi.nlm.nih.gov). The matched sequence was further analyzed using Peptide Mass tool, from www.expasy.org/tools.

3. Results and discussion

3.1. 1,3-β-D-glucanase production

Fungal 1,3-β-D-glucanase enzymes production is influenced by a number of factors including the type of strain used, the culture conditions and mainly the substrate type (Harman et al., 2004; Howell, 2003; Martin et al., 2007; Pitson, Martin, & McDougall, 1993). In order to evaluate the effect of different carbon sources on production of 1,3-β-D-glucanase by *T. asperellum*, the fungus were grown in TLE medium supplemented with glucose, starch, cellulose, chitosan, chitin and purified cell wall from *R. solani* (CWRS). No activity was detected in presence of 1% or 2% glucose, suggesting a regulation by catabolic repression. *T. asperellum* produced 1,3-β-D-glucanases in all polysaccharides tested but the levels of activity varied depending on the linkage type and structure of the carbohydrate used (Fig. 1A). Significant levels of 1,3-β-D-glucanase activity were found in presence of cellulose, chitosan and chitin, whereas highest activity was obtained with CWRS and starch (Fig. 1A). Whether any of these polymers or smaller saccharides derived from them is the true inducers for synthesis of these enzymes is unclear and needs to be studied. High levels of 1,3-β-D-glucanase activity in the culture containing CWRS, suggests that the secretion of these enzymes in *T. asperellum* was influenced by the level of β-glucan present in the cell walls of this phytopatogenic fungus. 1,3-β-D-glucanase activity was also found in *T. harzianum* cultures containing purified cell walls from *R.solani*, *S. rolfsii* and *Pythium* spp. (Noronha & Ulhoa, 2000).

To determine which secreted protein corresponded to 1,3-β-D-glucanases produced by *T. asperellum*, we assayed for enzyme activity by performing non-denaturing PAGE (Fig. 1B). No band of activity was detected with glucose as carbon source confirming the results obtained previously (Fig. 1A and 1B). One band was detected in starch, cellulose, chitosan or chitin (lines 3, 4, 5 and 6), whereas in presence of CWRS two bands with 1,3-β-D-glucanase activity were detected (line 7). The major and strong band of activity correspond to the 83.1 kDa exo-1,3-β-D-glucanase purified and characterized earlier by Bara et al. (2003). The minor band, corresponding to the 27 kDa 1,3-β-D-glucanase, was expressed only in presence of CWRS and depends of the concentration used (Fig. 2A and B). These data suggest that this enzyme may play an important role during the interaction between *T. asperellum* and *R. solani*.

3.2. Purification of the 1,3-β-D-glucanase

We have purified a minor 1,3-β-D-glucanase secreted by *T. asperellum* (Fig. 1B) using two step chromatography on Q-Sepharose and Sephacryl S-100 (Table 1). The purification procedure has a 70% yield and purification factor of 13.83 with a specific activity of 9.27 U mg^{-1} of protein. The purified

Table 1
Summary of the purification steps of the 27 kDa β-1,3-glucanase produced by *T. asperellum*.

Step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Purification (fold)	Yield (%)
Crude enzyme	44.25	0.198	0.67	1	100
Q-Sepharose	10.40	0.171	1.31	1.95	86
Sephacryl S-100	0.302	0.140	9.27	13.83	70

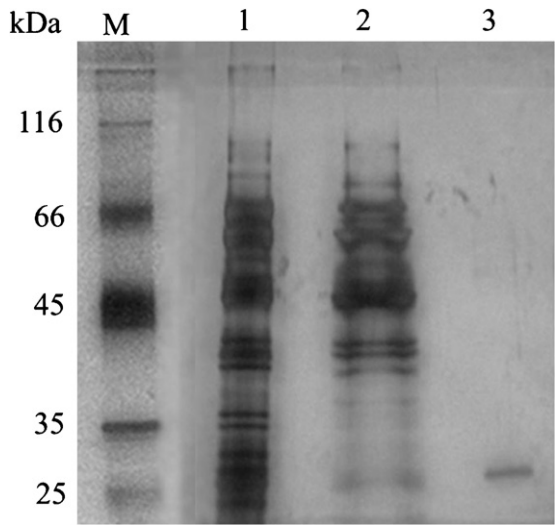

Fig. 3. SDS–PAGE profile of purified 1,3-β-glucanase. Electrophoresis was carried out using a 12% cross-linked polyacrylamide gel. Lane M, Molecular weight markers were β-galactosidase (116 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa) and REase Bsp98I (25 kDa); Lane 1, crude extract; lane 2, Q-Sepharose; lane 3, Sephacryl S-100. The amount of protein loaded was 30 μg.

1,3-β-D-glucanase appeared to be homogenous by analysis on SDS–PAGE, as shown in Fig. 3. The molecular weight of the enzyme was estimated to be approximately 27 kDa, based on its mobility calculated by comparison with standard calibration proteins using Quantity One software (Bio-Rad). The molecular weights of 1,3-β-D-glucanases produced by *Trichoderma* appear to vary considerably, not only between organisms, but also within the same species. 1,3-β-D-glucanases with molecular weight of 29, 31, 40 and 78 kDa were reported in *T. harzianum*, (De La Cruz, Pintor-Toro, Benitez, Llobell, & Romero, 1995; Dubourdieu, Desplanques, Villettaz, & Ribereau-Gayon, 1985; Kitamoto, Kono, Shimotori, Mori, & Ichikawa, 1987; Noronha & Ulhoa, 2000). In another study, we report the purification of an 83.1 kDa exo-1,3-β-D-glucanase produced by the same strain of *T. asperellum* (Bara et al., 2003).

3.3. Effect of pH and temperature

The effects of pH and temperature on the 27 kDa 1,3-β-D-glucanase activity are shown in Table 2. The optimum pH was 3.6, which is different to the 83.1 kDa exo-1,3-β-D-glucanase (Bara et al., 2003) and to that found from a variety of *T. harzianum* (El-Katatny, Gudelj, Robra, Elnaghy, & Gubitz, 2001; Kitamoto et al., 1987; Noronha & Ulhoa, 1996, 2000). The optimum pH of fungal 1,3-β-D-glucanase is usually in the range of 4.0–6.0 (Martin et al., 2007). The optimum temperature for the 1,3-β-D-glucanase was found to be 45 °C at pH 3.6 (Table 2). The enzyme was stable for at least 60 min when incubated at 45 °C, and retained 67% of maximum activity at 50 °C (Table 2). This enzyme is more stable than the 83.1-kDa exo-1,3-β-D-glucanase that retained only 85% and 57% of the original activity after incubation for 60 min at 45 °C and 50 °C, respectively (Bara et al., 2003).

Table 2
Biochemical properties of the purified 27 kDa β-1,3-glucanase from *T. asperellum*.

Molecular weight (kDa)	27 ± 2.0
pH optimum	3.6 ± 0.2
Temperature optimum (°C)	45 ± 1.0
Temperature stability	
pH 3.6/45 °C/60 min	100% ± 2%
pH 3.6/50 °C/60 min	57% ± 2%
K_m (mg ml^{-1})	0.323
V_{max} (U min^{-1})	0.315
Inhibition by Hg^{2+}	100% ± 1.0%
Inhibition by SDS	52% ± 2.0

The molecular weights of the enzymes were determined by gel SDS–PAGE. The effect of pH on the enzyme activity was determined by varying the pH of the reaction mixtures using 100 mM citrate/phosphate buffer (pH 2.0–7.0). The effect of temperature on the enzymatic activity was determined at the pH 3.6 in the range of 25–60 °C. The effect of temperature on the enzyme stability was analyzed by previously incubating the enzyme at 45and 50 °C for 60 min. Kinetics parameters of the purified β-1,3-glucanase were estimated for laminarin by using a concentration ranging from 50 to 300 μg ml^{-1}. The effects of $HgCl_2$ and SDS on β-1,3-glucanase activity were determined after pre-incubation with the respective compound (2.5 mM) for 5 min at 45 °C. Results are means values of three replicates.

Table 3
Substrate specificity of endo-β-1,3-glucanase from *T. asperellum*.

Substrate (0.5%)	Main linkage/monomer	Relative activity (%)
Laminarin	β-1,3-/glucose	100
CWRS	β-1,4-; β-1,3-/GlcNAc; glucose	84.3
Cellulose	β-1,4-/glucose	0
Chitin	β-1,4-/GlcNAc	0
Chitosan	β-1,4-/GlcN	0
Starch	β-1,4-; β-1,6/glucose	0

The enzyme activity was expressed as a percentage with respect to activity determined on laminarin. Results are means values of three replicates. CWRS: purified cell wall from *R. solani*.

3.4. Substrate specificity and kinetics constants

To establish the specificity of 27 kDa 1,3-β-D-glucanases against a variety of glucan substrates, laminarin, CWRS, cellulose, chitin, chitosan, and starch were used as substrates (Table 3). The purified enzyme was active only toward glucans containing β-1,3-linkages, such as laminarin, and CWRS (Table 3). The enzyme hydrolyzed laminarin more readily than purified cell wall, but did not attack cellulose, chitin, chitosan and starch. Inactivity of the enzyme toward cellulose suggested that the enzyme is unable to cleave β-1,4-linkages within the β-glucan molecule. The affinity and specificity for different substrates may be related to physiological functions performed by these enzymes in these organisms. The apparent K_m and V_{max} values for laminarin were 0.323 mg ml^{-1} and 0.315 U min^{-1}, respectively (Table 3). This K_m value was lower than those previously reported for 36 kDa 1,3-β-D-glucanases (1.18 mg ml^{-1}) (23 Noronha & Ulhoa, 1996), the 29 kDa 1,3-β-D-glucanase (1.72 mg ml^{-1}) (Noronha & Ulhoa, 2000) and the 70 kDa 1,3-β-D-glucanase (3.3 mg ml^{-1}) (De La Cruz et al., 1995) produced by *T. harzianum*. However, the K_m value is higher than that found for the 83.1 kDa exo-1,3-β-D-glucanase produced by the same strain of *T. asperellum* (0.087 mg ml^{-1}) (Bara et al., 2003).

The denaturing agents SDS and Hg^{2+} strongly inhibited the enzyme activity (Table 2). The complete inhibition by mercuric ions may indicate the importance of sulfhydryl groups in enzyme functions, as reported for purified 1,3-β-D-glucanase from *T. harzianum* (TC) and *T. longibrachiatum* (Lorito, Hayes, Dipietro, Woo, & Harman, 1994; Noronha & Ulhoa, 2000).

3.5. Protein Identification

The minor 1,3-β-D-glucanase was digested with trypsin and the peptides were analyzed in a MALDI-TOF/TOF. The MS spectra obtained was analyzed and then monoisotopic peptides masses were selected and fragmented. The MS/MS spectra obtained was manually analyzed and two peptides could be sequenced/identified (Table 4). The residues Leu/Ile and Gln/Lys that could not be distinguished by means of the MALDI-TOF/TOF *tandem* mass spetrometric data, whenever possible, were suggested based upon

Table 4
Amino acid sequence and protein identification from MS/MS spectra.

Identified protein	Accession number[a]	Organism	Peptide sequence[b]	*e*-Value	Theoretical MW[c]/p*I*
Endo-1,3(4)-beta-glucanase	XP_751328.1	*Aspergillus fumigatus* Af293	IESTYTFTPGAGKVTAVEASIR	1.00E−05	31,087/5.09
Endo-1,3(4)-beta-glucanase	XP_748630.1	*Aspergillus fumigatus* Af293	TPSSWQSETITWSLDGTNYFQITGSR	1.00E−06	30,669/4.79

[a] Accession number from GenBank (NCBI).
[b] Peptide sequence obtained through manual *de novo* interpretation of MS/MS spectra.
[c] Molecular weight expressed in Da.

similarity with the most homologous proteins sequences from the database.

The peptides sequenced were 22 and 26 amino acids residues long each and matched to two endo-1,3(4)-β-D-glucanases from *Aspergillus fumigatus* Af293, both 285 amino acids long, but with some differences in its residues. The peptide IESTYTFTPGAGKVTAVEASIR best matched an endo-1,3(4)-β-D-glucanases with a theoretical molecular weight of 31 kDa and p*I* 5.09, while the peptide TPSSWQSETITWSLDGTNYFQITGSR matched with an 30.7 kDa/4.79 endo-1,3(4)-β-D-glucanases. The homology of peptide sequences indicates that the purified enzyme is a low molecular weight 1,3-β-D-glucanase, although it did not show activity in glucans containing β-1,4-linkage as the homologous proteins from *Aspergillus fumigatus* Af293 suggests. The lack of a published genome is a big issue to identify homologous proteins accurately and can lead to misinterpretations. For that reason, manual interpretation of MS/MS spectra is a good option to overcome possible mistakes in peptide sequence when automatically done and, therefore, in protein identification.

4. Conclusions

T. asperellum produces two proteins with 1,3-β-D-glucanases activity when induced with cell wall isolated from *R. solani*. A minor extracellular 1,3-β-D-glucanases from *T. asperellum* was purified approximately 13.8-fold with an overall yield of 70% and specific activity of 9.27 U mg^{-1}. It is a single peptide chain with a molecular weight of 27 kDa. This enzyme showed different physicochemical characteristics when compared with the 83.1 kDa exo-1,3-β-D-glucanases purified from the same strain of *T. asperellum* and showed similar sequences with two endo-1,3(4)-β-D-glucanases produced by *Aspergillus fumigatus* Af 293. Further studies will be required to determine the role of these enzymes during the interaction between *T. asperellum* and *R. solani*.

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