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Purification and Characterization of a β-Glucanase Produced by *Trichoderma harzianum* Showing Biocontrol Potential

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ABSTRACT

A β-1,3-glucanase was produced by Trichoderma harzianum *in cultures containing chitin as the sole substrate. Two proteins showing β-1,3-glucanase activity were purified to apparent homogeneity by hydrophobic chromatography. The molecular masses of these proteins were 29 and 36 kDa. The 36 kDa protein was further characterized. It was active on a broad pH range, and maximal activity was detected at pH 5.0. The optimum temperature of the 36 kDa β-1,3-glucanase was 50°C, but the purified enzyme was very sensitive to temperature. It lost about 60% or more of the activity after incubation for 30 min at 45, 50 and 60°C. The apparent K_M and V_{max} for hydrolysis of laminarin at pH 5.0 and 37°C, were 0.099 mg of reducing sugar/mL and 0.3 mg of reducing sugar/min.mL, respectively. The enzyme was insensitive to organic compound and metal ions, except for the ferric ion which inhibited about 100% of the original activity at the concentration of 1 mM. In contrast to other hydrolytic enzymes (a chitinase and a protease) produced by the same* T. harzianum *isolate (1051), the β-1,3-glucanase showed no effect on the cell wall of the phytopathogenic fungus Crinipellis perniciosa.*

Key words: *Trichoderma harzianum* , β-1,3-glucanase, chitinase, proteases

INTRODUCTION

A linear 1,3-1,4-β-glucan which accounts for up to 5.5% of the dry weight of grains is the major cell wall polysaccharide in the endosperm of cereals (McCleary, 1988) The bulk of the polysaccharide consists of short regions of β-1,4- linked glucose residues (three to four) interrupted by a single β-1,3-linkage (Dais and Perlin, 1982). Cereals have a high water-binding capacity and form highly viscous solutions and gelatinous suspensions in aqueous media. Consequently, the brewing industries faces problems such as increased worth viscosity, longer process times for beer filtration and β-glucan precipitation in beer (Bamforth and Quain, 1990) In addition, excess cell wall β-glucan impairs malt extraction, leading to prolonged malting times and a decrease in the brewhouse yield (Esslinge et al., 1985). The presence of cereal β-glucan in poultry and animal diet is similarly problematic in that "gummy" and indigestible β-glucans have inconsistent nutritional values, contribute to wet and sticky faeces and depress animal performance (Jeroch et al., 1988). Therefore, enzymes that catalyze depolymerization of selective modification of cereal β-glucans are of biotechnological and biomedical importance (McCleary et al., 1988). β-glucan-degrading enzymes are produced by a wide variety of organisms. Several types of these enzymes exist,

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classified according to the type of β-glucosidic linkage that they cleave and the mechanism of substrate attack. They can hydrolyze the substrate by two possible mechanisms, identified by the products of hydrolysis: (*i*) exo-β-glucanase hydrolyze the substrate by sequentially cleaving glucose residues from the nonreducing end, or (*ii*) endo-β-glucanases cleave β-linkage at random sites along the polysaccharide chain, releasing smaller oligosaccharides. Degradation of β-glucan by fungi is often accomplished by the synergistic action of both endo- and exo-β-glucanases (Pitson et al., 1993).

On the other hand, the cell walls of the plant pathogens *Sclerotium rolfsii*, *Rhizoctonia solani*, and *Pythium* sp (Chet, 1987) are largelly composed of 1,3-β-glucans and chitin, with cellulose also found in Oomycetes, e.g., *Pythium* spp. (Bartinicki-Garcia, 1973)**.** In recent years several works have suggested that chitinase and β-glucanase producing fungi, i.e., species of *Trichoderma*, could be effective as biological control agents. Micoparasitism has been described as the main process involved in the antagonistic action of *Trichoderma harzianum* against fungal pathogens. It is a complex process that includes the release of lytic enzymes by the *Trichoderma*, degradation of the cell wall (Lima et al., 1997), and further penetration in the host mycelium (Chérif and Benhamou, 1990). Two *Trichoderma* isolates (1051 and TVC), previously found to control witches' broom of cocoa under field conditions (José Luiz Bezerra, personal communication), produced substantial amounts of chitinase, N-acetylglucosaminidases, β-glucanases, proteases, cellulases and amylases in cultures containing the corresponding substrates (De Marco et al., 2003). Studies have been reported on the purification and characterization of one chitinase (De Marco et al., 2000), one protease (De Marco and Felix, 2002), one amylase (Azevedo et al., 2000), and one N-acetylglucosaminidase (De Marco et al., 2004) produced by the isolate 1051. While the chitinase strongly attacked the *Crinipellis perniciosa* cell wall, the protease and the N-acetylglucosaminidase were less effective, and the amylase had no effect at all. Here, we report on the purification and characterization of a β-glucanase produced by *T. harzianum* 1051 growing in liquid medium containing chitin as the sole carbon source, and on the lack of action of this enzyme on the *C. perniciosa* cell wall.

MATERIALS AND METHODS

Strain origin and maintenance

Trichoderma harzianum, isolate 1051, was obtained from the collection of the Centro Nacional de Pesquisa de Monitoramento e Avaliação de Impacto Ambiental (CNPMA/EMBRAPA, Jaguariuna, SP, Brazil –the National Research Center for Monitoring and Environmental Impact Assessment). The phytopathogen *C. perniciosa* was kindly provided by Dr. José Luiz Bezerra from CEPLAC/Ilheus, BA, Brazil. Both microorganisms were maintained by serial passages on potato-dextrose agar medium (Difco).

Enzyme production and assay

For enzyme production, *T. harzianum* spores (2 x 10^6) were inoculated in 250 mL TLE liquid medium (0.1% bactopeptone; 0.03% urea; 0.2% KH_2PO_4; 0.14% $(NH_4)_2SO_4$; 0.03% $MgSO_4 \cdot 7H_2O$; 0.03% $CaCl_2 \cdot 6H_2O$; 1 mL of 0.01% trace elements solution (Fe^{2+}, Mn^{2+}, Zn^{2+}, and Co^{2+}), 0.02% glucose, pH 5.5) containing chitin (0.5%), for 72h at 28^{o}C. The cultures were incubated for 0-72h at 28^{o}C, with agitation (250 rpm). The culture supernatants were separated from the mycelium by filtration through filter paper, and used for enzyme assay and/or enzyme purification as described in the section below.

β-glucanase activity was assayed (in triplicate) using a reaction system containing 250 μL of a laminarin solution (1%) dissolved in 50 mM sodium acetate buffer, pH.5.0 and 0-125 μL of enzyme solution. Reaction was allowed to proceed for 30 min at 37^{o}C and stopped by addition of 1.5 mL of 3,5-dinitrosalicylic acid reagent. The reducing sugar formed was then determined spectrophotometrically at 550 nm according to Miller (1959). One unit of enzyme (U) was defined as the amount of protein necessary to produce one micromole of reducing sugar per min. For determination of optimum pH, the assays were run for 30 min at 37^{o}C, using the buffer solutions (50 mM) of sodium acetate (pH 3.0 – 5.0), sodium phosphate (pH 6.0 – 8.0) and Tris-HCl (pH 9.0) containing the substrate laminarin. In all cases, the standard deviation values for enzyme activity were

smaller than 5% of the mean values. For determination of K_M and V_{max} of laminarin hydrolysis, the concentrations of this substrate varied from 0.1 mg to 20.0 mg/ mL.

Enzyme purification

To the supernatants from *T. harzianum* 1051 cultures obtained as described above, $(NH_4)_2SO_4$ was added to a concentration of 40%. The mixtures were kept for 15 min at 4°C under gentle agitation. Suspensions were centrifuged at 28,800 g for 30 min, and the pellets were discarded. Ammonium sulfate was then added to supernatants for a final concentration of 60%. After 15 min at 4°C under gentle agitation, the supernatants were centrifuged as above, and the resulting pellets resuspended in a small volume of 50 mM sodium acetate pH 5.0, and used as crude enzyme preparation. Samples of crude enzyme were then loaded on a Phenyl Sepharose CL 4B column (10 x 1.8 cm) equilibrated with acetate buffer 50 mM, pH 5.0. Proteins were then eluted with a negative gradient formed with 40 mL of the acetate buffer containing 1M ammonium sulfate, and 40 mL of the acetate buffer. Samples of 2.6 mL were collected at a flow rate of 52 mL/h. Protein (A_{280nm}) and β-glucanase activity were monitored for each sample. Fractions containing the enzyme were pooled, dialyzed against distilled water, and concentrated by freeze-drying.

The concentration of protein in the enzyme samples was determined by a simplification of the method of Lowry et al. (Peterson, 1977).

SDS-PAGE analysis

β-glucanase samples were analysed by electrophoresis in polyacrylamide gels under denaturing conditions, according to Laemmli (1970). Proteins present in the gels were stained with silver reagent according to the method of Blum et al. (1987).

Enzymatic digestion of *Crinipellis perniciosa* mycelium and scanning electron microscopy (SEM) analysis

Disks (5 mm) of solid culture medium containing *C. perniciosa* mycelium were placed in microcentrifuge tubes containing 1 mL of either *T. harzianum* 1051 culture supernatant showing β-glucanase activity (2.2 U/mL), or the corresponding purified enzyme (0.47 U/mL). After incubation for 96h at 37°C, the supernatants were used for determination of reducing sugars by the dinitrosalicylic acid method (DNS assay) according to Miller (1959), glucose by the glucose oxidase method (glucose-oxidase assay, Doles Reagents kit, Goiania, GO, Brazil) and protein by a simplification of the method of Lowry et al.. (Peterson, 1977). The *C. perniciosa* mycelium disks, treated or without β-glucanase, were then fixed for 2h at 4°C in 2% (v/v) glutaraldehyde and 2% (w/v) paraformaldehyde, buffered with 0.05% sodium cacodylate buffer, pH 7.2, and postfixed in 1% (w/v) osmium tetroxide in the same buffer. The specimens were washed with buffer and dehydrated in 30-100% (v/v) acetone, critical-point dried in CO_2 and sputter-coated with gold. Materials were examined microscopically at an accelerating voltage of 10.0 kV. Disks of solid medium containing *C. perniciosa* treated with heat denatured β-glucanase were used as a control.

RESULTS AND DISCUSSION

Production of β-1,3-glucanase

The production of extracellular β-glucanase was monitored during growth of *T. harzianum* isolate 1051 growing in TLE liquid medium supplemented with 0.5% chitin. As shown in Fig. 1, the enzyme activity present in the culture supernatant increased rapidly after 24h, and a higher enzyme level was detected with 72h of growth. This enzyme was considered to be a β-1,3-glucanase, or laminarinase (1,3-glucan-3-glucanohydrolase; EC 3.2.1.39), since it hydrolysed laminarin, a poly β(1→3)-glucan, a substrate for laminarinase. β-glucanases are produced and secreted into the culture medium by a wide variety of fungi in response to the presence of chitin (Noronha and Ulhoa, 2000; Pitson et al., 1993). Frequently, in nature, *Trichoderma* β-1,3-glucanases are responsible for hydrolysis of phytopathogenic fungi during a mycoparasite attack (Herrera-Estrella and Chet, 1998). In fact, β-1,3-glucanase activity was also found in cultures containing purified cell walls from *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium* spp. as inducer (Noronha and Ulhoa, 2000). In this case, the enzyme activity was higher in the culture containing *Pythium* spp. cell wall, suggesting that secretion of β-1,3-glucanases was greatly influenced by the levels of β-glucan present in the cell walls of this phytopathogenic fungus. The

possible action of the β-1,3-glucanase produced by the *Trichoderma* isolate 1051, on cereal β-glucans, which caused problems in brewing industry and which affected the performance of poultry and animal diets (Murray et al., 2001) could be an opening for additional investigation.

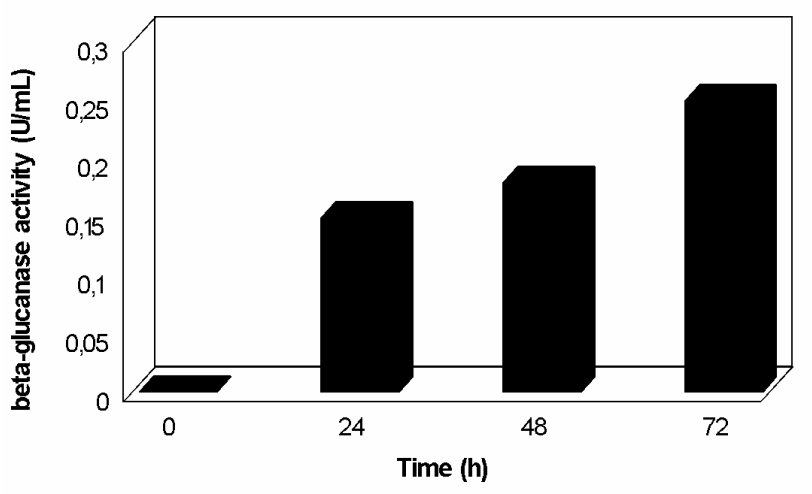

Figure 1 - Time course of β-1,3-glucanases produced by *T. harzianum* 1051 in liquid medium containing chitin (0.5%), under agitation (130 rpm) at 28°C.

Purification of the β-1,3-glucanase

The proteins showing β-1,3-glucanase activity were separated by fractionated precipitation with ammonium sulfate. In the presence of 40% of ammonium sulfate, very low, or no β-1,3-glucanase was precipitated. However, the bulk of the enzyme present in solution was removed with 60% of the ammonium salt. The protein present in the resulting pellet was easily fractionated by hydrophobic chromatography in a Phenyl Sepharose CL 4B column. Relevant β-1,3-glucanase activity was eluted in two major overlapping peaks (Fig. 2). SDS-polyacrilamide gel electrophoresis revealed that peak number one contained a single protein having β-1,3-glucanase activity and a molecular mass of 29 kDa; and the second peak, a β-1,3-glucanase of molecular mass of 36 kDa (Fig. 3). It was previously reported that *T. harzianum* produced three β-1,3-glucanases in the presence of chitin as carbon source. One of these enzymes was purified and characterized as an endo-β-1,3-glucanase with a molecular mass of 36 kDa (Noronha and Ulhoa, 1996). Another β-1,3-glucanase produced by *T. harzianum* (Tc) also showed a molecular mass of 36 kDa (Noronha and Ulhoa, 2000). Although different approaches were used to purify these enzymes, it was likely that the two β-1,3-glucanases (29 kDa and 36 kDa) reported here and those reported by were Noronha and Ulhoa (1996; 2000) are the same protein. While low molecular weight β-1,3-glucanases were also present in phytopathogenic fungi like *Rhizoctonia solani* (Totsuka and Usui, 1986), a high molecular mass extracellular endo-β-1,3-glucanase was produced by the mycoparasitic fungus *T. harzianum* (CECT 2413). The gene encoding this enzyme was cloned and found to encode a protein of a molecular mass of 78 kDa (De La Cruz et al., 1995).

Optimal pH and temperature, and stability of the 36 kDa β-1,3-glucanase

The purified 36 kDa β-1,3-glucanase displayed activity over a broad pH range, i.e., activity was detected at pH from 3-9 at the temperature of 37°C. However, maximal activity was measured at pH 5.0, and decreasing activity was detected when the enzyme assays were performed in conditions having lower, or higher pH values (Fig. 4A).

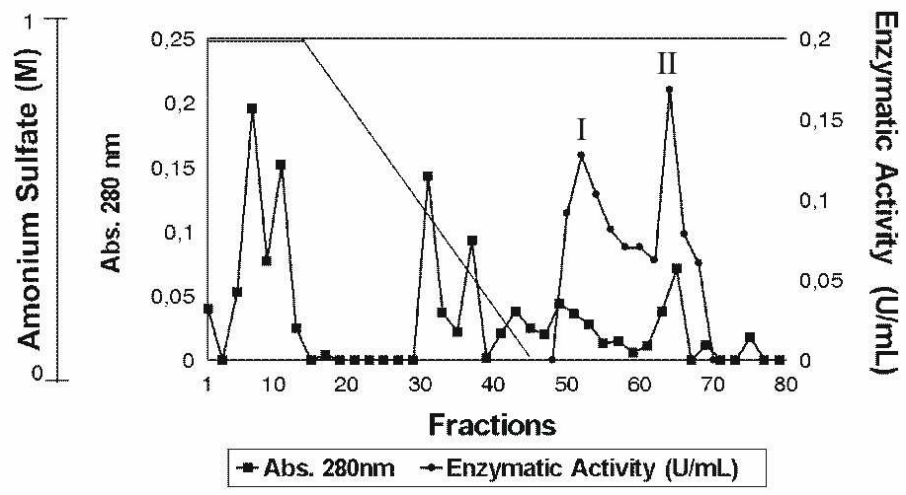

Figure 2 - Phenyl Sepharose CL 4B hydrophobic chromatography (10 x 1.8 cm column) of the culture filtrate of *T. harzianum* 1051 grown in the presence of chitin (see fig. 1). The culture supernatant was submitted to precipitation with ammonium sulfate as described in material and methods. The diagonal line indicates the ammonium sulfate gradient formed with 0 to 1M.

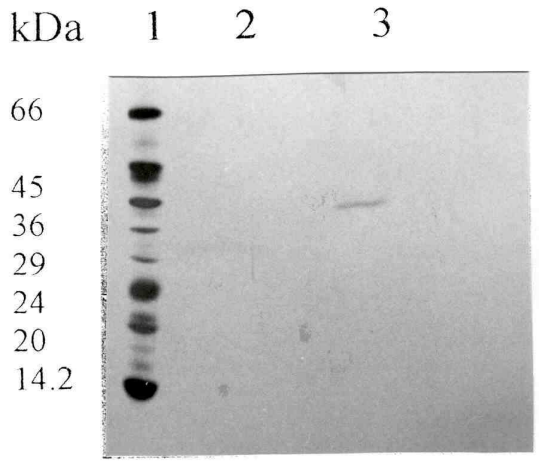

Figure 3 - SDS-PAGE of the β-1,3-glucanase from *T. harzianum* 1051 purified on a Phenyl-Sepharose column. **1**, molecular weight standards; **2**, 29 kDa β-1,3-glucanase purified; **3**, 36 kDa purified β-1,3-glucanase.

Substantial enzyme activity could be measured into a temperature range of 20–80°C. The optimum temperature for this enzyme was 50°C (Fig.4B). The optimal pH and temperature values found for 36 kDa β-1,3-glucanase was very close or the same as those calculated for the 29 kDa (pH 4.4, and temp. 50°C) and the 36 kDa (pH 4.4, and temp. 45-50°C) β-1,3-glucanases reported by Noronha and Ulhoa (1996; 2000). However, 36 kDa purified β-1,3-glucanase was not resistant to temperature. At temperatures varying from 45°C to 60°C, the 36 kDa enzyme lost almost all its activity within 30 min (Fig. 4C).

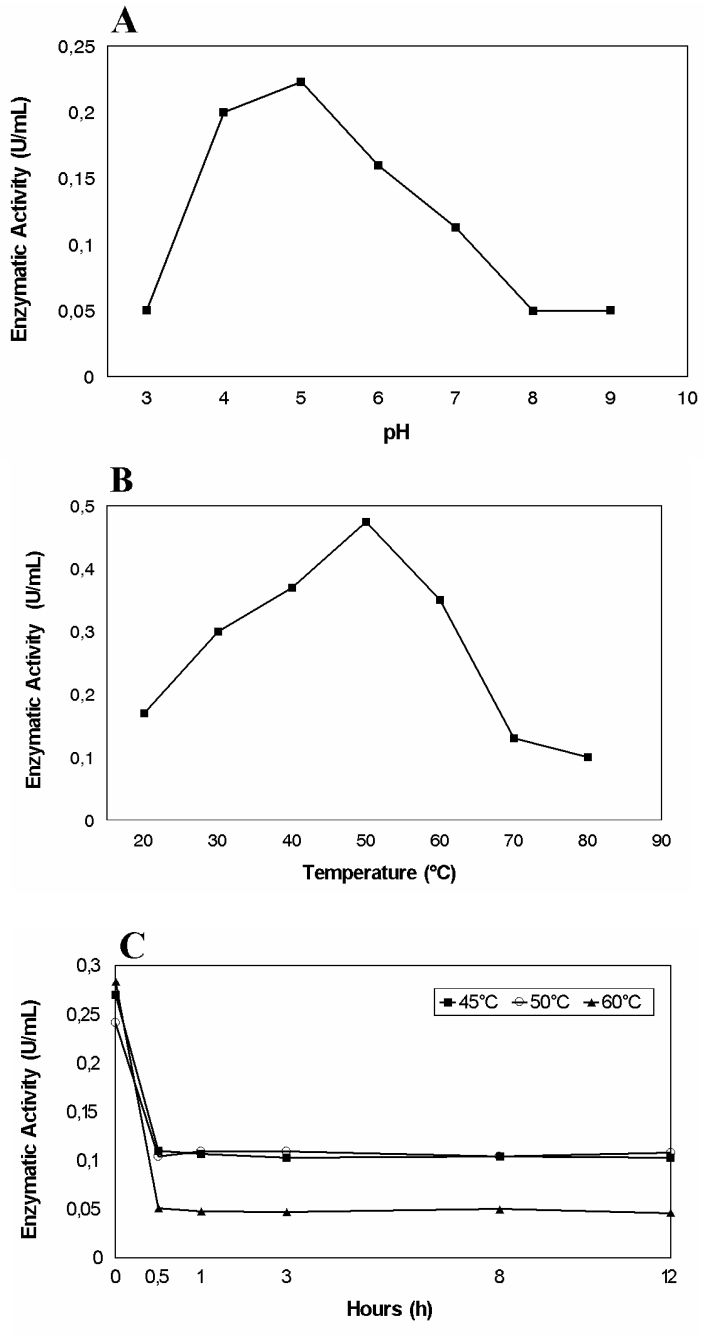

Figure 4 - pH **(A)** [the effect of pH was determined by varying the pH of the reaction mixtures by using 50mM sodium acetate (pH 3.0-5.0), 50mM sodium fosfate (pH 6.0-8.0), 50mM Tris HCl (pH 9.0)], temperature **(B)**, and thermostability **(C)** profiles of the purified 36 kDa β-1,3-glucanase from *T. harzianum* 1051.

This low thermostability was in agreement with the data found by Noronha and Ulhoa for the 29 kDa β-1,3-glucanase (Noronha and Ulhoa, 2000) but not for the 36 kDa β-1,3-glucanase (Noronha and Ulhoa, 1996), which was found to be fairly resistant to temperature. Nevertheless, these values reported above were much lower than the optimal temperatures reported for β-glucanase produced by extremophilic microorganisms. A lichenase from the anaerobic thermophilic bacterium *Clostridium thermocellum* was reported to have an optimum temperature of 80°C

(Schwimming et al., 1991). A purified endoglucanase active on 1,3-1,4-β-D-glucans produced by the moderately thermophilic aerobic fungus *Talaromyces emersonii* CBS 814.70 displayed activity over a broad range of pH and temperature, yielding optimal values of pH 4.8 and 80°C. This enzyme was markedly thermostable with 15% of its original activity remaining after incubation for 15 min at 100°C (Murray et al., 2001). Although the enzyme was composed of a single protein with a molecular mass of 40.7 kDa, its carbohydrate content was estimated to be 77% (w/w). The 35.9 kDa endo-1,4-β-glucanase from the thermophilic bacterium *Pyrococcus furiosus* had a temperature optimum of 100°C, and the $t_{1/2}$ at 95°C was 40h. The denaturing temperature reported for this enzyme was 112°C (Bauer et al., 1999).

Kinetics of substrate hydrolysis and the effect of ion on the 36 kDa β-1,3-glucanase

The effect of laminarin on the activity of the purified 36 kDa β-1,3-glucanase from *T. harzianum* 1051 was analyzed. At 37°C and pH 5.0, the enzyme hydrolyzed laminarin with a Michaelis-Menten kinetic (not shown). The K_M (0.099 mg/mL) and V_{max} (0.3 mg/min.mL) values for laminarin hydrolysis were determined using a non-linear regression data analysis program (Curve expert program 1.24 for Windows)**.** This K_M value was lower than those previously reported for laminarin hydrolysis by the previously reported 36 kDa β-1,3-glucanase (1.18 mg/mL) (Noronha and Ulhoa, 1996), the 29 kDa β-1,3-glucanase (1.72 mg/mL) (Noronha and Ulhoa, 2000) and the 70 kDa β-1,3-glucanase (3.3 mg/mL) (De La Cruz et al., 1995) produced by *T. harzianum*.

The effects of metal ions and organic compounds on the activity of the 36 kDa β-1,3-glucanase from *T. harzianum* are shown in Table 1. Except for the $FeCl_3$, which showed a markedly inhibitory effect (98.6%), all the ions tested were ineffective and/or showed a very low effect. The detergent SDS and the reducing agent β-mercaptoethanol did not drastically affect the enzyme.

Table 1 - The effect of ions on the activity of the purified β-1,3-glucanase from *T. harzianum* 1051.

Reagents	Enzymatic activity (U/mL)	Relative activity (%)
Control	4.89 ± 0.009	100
SDS (1mM)	4.38 ± 0.02	89.4
β-mercapt. (1mM)	4.6 ± 0.019	94
$FeCl_3$ (1mM)	0.072 ± 0	1.4
$MgSO_4$ (1mM)	4.07 ± 0.0028	83
$MgSO_4$ (5mM)	4.19 ± 0.043	86
$AlCl_3$ (1mM)	4.56 ± 0.004	93
$AlCl_3$ (5mM)	5.66 ± 0.007	115
$ZnSO_4$ (1mM)	4.45 ± 0.002	91
$ZnSO_4$ (5mM)	4.5 ± 0.005	92
$CaCl_2$ (1mM)	4.75 ± 0.018	97
$CaCl_2$ (5mM)	5.3 ± 0.011	108

Action of the 36 kDa β-1,3-glucanase on the *Crinipellis perniciosa* cell wall

Incubation of the mycelium of *C. perniciosa* with the crude extract containing β-1,3-glucanase activity did not result in the production of reducing sugars, or glucose (data not shown). According to the electron microscopy analysis of the *C. perniciosa* mycelium treated or without the crude, or the purified β-1,3-glucanase, no evident effect occurred in the mycelium (figures not shown). These findings were in contrast with the results reported for treatment of the *C. perniciosa* with a 37 kDa chitinase (De Marco et al., 2000), 18.8 kDa protease (De Marco and Felix, 2002) and a 36 kDa N-acetylglucosaminidase (De Marco et al., 2004) produced by the same isolate (1051) of *T. harzianum.* Research on the possible mechanisms involved in biological control by *Trichoderma* species has led to several alternative explanations for successful biocontrol (Howell, 2003). In brief, enzymes such as chitinases and/or glucanases produced by the biocontrol agent are responsible for suppression of the plant pathogen. These enzymes would catalyse the break down of the

polysaccharides chitin and β-glucans destroying the cell integrity. It could be also suggested that proteases produced by the mycoparasitic fungus would hydrolysed the enzymes produced by the phytopathogen (Elad and Kapt, 1999). Transformants of *T. longibrachiatum* (CECT2606) over-expressing a gene encoding β-1,4-endoglucanase, were slightly more effective in the biocontrol of *Pythium ultimum*. However, it was also concluded that a mixture of enzymes might be necessary for efficient cell wall lysis (Migheli et al., 1998). The possibility that the β-1,3-glucanase reported here acted *in vivo* on the *C. perniciosa* cell wall only in combination with others micolytic enzymes was so far not discarded.

RESUMO

Uma β-1,3-glucanase foi produzida por *Trichoderma harzianum* em cultura contendo quitina como fonte de carbono. Duas proteínas com atividade de β-1,3-glucanase foram purificadas através de cromatografia de interação hidrofóbica. As massas moleculares destas proteínas foram de 29 kDa e 36 kDa. A proteína de 36 kDa foi caracterizada quanto à influência das condições de pH e temperatura. A atividade máxima foi encontrada em pH 5,0 e temperatura de 50^{o}C. A proteína purificada mostrou-se muito sensível à temperatura. Aproximadamente 60% da atividade original foi perdida por incubação da proteína a 45^{o}C, 50^{o}C e 60^{o}C, por 30 min. O K_M aparente e a V_{max} para hidrólise de laminarina em pH 5,0 à 37^{o}C, foram de 0,099 mg de açúcar redutor/mL e 0,3 mg de açúcar redutor/min.mL, respectivamente. Esta enzima mostrou-se insensível a compostos orgânicos e íons metálicos, exceto íon férrico o qual em uma concentração de 1 mM, inibiu em aproximadamente 100% a atividade da enzima. Ao contrário de outras enzimas hidrolíticas (quitinase e protease) produzidas pelo mesmo isolado 1051 de *T. harzianum*, a β-1,3-glucanase descrita aqui não afetou a integridade da parede celular do fitopatógeno *Crinipellis perniciosa.*

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