Biotechnol Lett (2007) 29:617–622
DOI 10.1007/s10529-006-9287-0

ORIGINAL RESEARCH PAPER

Purification and characterization of a β-1,3-glucanase from the novel mycoparasite *Periconia byssoides*

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Received: 22 September 2006 / Revised: 29 November 2006 / Accepted: 4 December 2006 /
Published online: 23 January 2007

Abstract An extracellular β-1,3-glucanase with antifungal properties was secreted by the novel mycoparasite, *Periconia byssoides*. The glucanase has a molecular mass of 35 kDa estimated by SDS-PAGE. Its optimum activity was at pH 6.0 and 50°C (over 2 h). The purified β-1,3-glucanase was capable of degrading cell walls, and inhibiting mycelia growth and spore germination of plant pathogenic fungi including *Fulvia fulva*, *Fusarium* sp. and *Rhizoctonia solani*. The *N*-terminal amino acid residues of the purified β-1,3-glucanase are LKNGGPSFGA, which do not have any homology with previously described glucanases, suggesting it may be a novel member of the fungal β-1,3-glucanases.

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Keywords Antifungal activity · β-1,3-glucanase · Mycoparasite · *Periconia byssoides* · Purification

Introduction

Mycoparasites are fungi that can parasitize the mycelia of other fungi (Li et al. 2005). They could be used as an attractive biological alternative to the strong dependence of modern agriculture on chemical fungicides (Leroux 2003). A typical fungal cell wall is composed of chitin, glucans and proteins and can be degraded by chitinases and glucanases, which are produced by several mycoparasites (Schirmbock et al. 1994; Perez et al. 2002). *Trichoderma* is one of the best known and well described biocontrol fungi and has been studied for a long time for its lytic activity (Rey et al. 2001) and antagonistic properties against plant pathogenic fungi (Rocha-Ramírez et al. 2002). Glucanases are widely distributing among mycoparasites and some of them have been purified. In addition, several glucanase-associated genes have been cloned and studied via expression experiments (Palumbo et al. 2003; Nobe et al. 2004). Glucanases seem to have multiple functions as some of them have a nutritional role in mycoparasites (Donzelli and Harman 2001).

Periconia spp. have been isolated widely from biological samples and research focuses mainly on the biological active metabolites produced by this

species such as the cell-adhesion inhibitors produced by *P. byssoides* (Yamada et al. 2005), the stereostructure of *seco*-macrosphelide E produced by *P. byssoides* (Nakamura et al. 2002) and some toxins/peritoxins with host-selective toxicity produced by *P. circinata* (Macko et al. 1992; Churchill et al. 2001). However, ongoing research with *Periconia* has not reported any species that can parasitize other fungi and/or the ability to produce enzymes involved in mycoparasitism.

In this study, a novel mycoparasite *Periconia byssoides* was identified that can parasitize plant pathogenic fungi: *Fulvia fulva*, *Fusarium* sp. and *Rhizoctonia solani*. Moreover, the purification, biochemical characterization and antifungal activity of an extracellular β-1,3-glucanase secreted by *P. byssoides* was studied, its N-terminal amino acid residues and possible role of this enzyme in mycoparasitism are discussed.

Materials and methods

Strains and culture condition

Fulvia fulva, *Fusarium* sp., *Periconia byssoides* and *Rhizoctonia solani* were obtained from the culture collection of the Laboratory for Conversation and Utilization of Bio-resources, Yunnan University, and maintained on potato/dextrose/agar (PDA) at 27°C. The medium (CM) for production of β-1,3-glucanase by *P. byssoides* contained: 2 g corn-meal l^{-1}, 1 g $(NH_4)_2SO_4$ l^{-1}, 1 g KH_2PO_4 l^{-1}, 0.25 g $MgSO_4 \cdot 7H_2O$ l^{-1}, 0.25 g NaCl l^{-1}, 10 mg $CaCl_2$ l^{-1}, 5 mg $FeSO_4$ l^{-1}, 5 mg $ZnSO_4$ l^{-1}, pH 6.0 adjusted with 0.5 M NaOH. *P. byssoides* was cultivated in CM medium for 3 days at 27°C with shaking at 200 rpm.

Dual-culture plate assay

To study the possible interactions between *P. byssoides* and the plant pathogenic fungi *F. fulva*, *Fusarium* sp. and *R. solani*, *P. byssoides* and plant pathogenic fungi were inoculated on the surface of a water agar (2% w/v) plate (9 cm), and they were incubated on opposite each other (6 cm) (Sun et al. 2006). The interactions between *P. byssoides* and the plant pathogenic fungi were examined under a light microscope after culturing for 3–15 days at 27°C.

Protein determination and enzyme analysis

Protein concentration was determined by the method of Bradford using BSA as a standard. The enzyme activity of the purified β-1,3-glucanase was measured with laminarin (from *Laminaria digitata*, Sigma) as substrate (Miller 1959). Laminarin was dissolved in 0.1 M NaH_2PO_4/NaOH buffer (pH 6.0). The reaction mixture containing 1 ml 0.2% laminarin and 1 ml enzyme solution was incubated at 50°C for 2 h. The reaction was terminated by adding 2 ml of 1% dinitrosalicylic acid and boiled for 5 min. The amount of reducing saccharides released from laminarin was measured spectrophotometrically at 540 nm. One unit (U) of β-1,3-glucanase activity was defined as the amount of the enzyme that released reducing saccharides equivalent to 1 μg glucose per minute under the above conditions.

SDS-PAGE and *N*-terminal amino acid sequence analysis

SDS-PAGE on 12% (w/v) polyacrylamide slab gels was carried out according to Laemmli (1970). Gels were stained with Coomassie Brilliant Blue and destained with an aqueous mixture of 10% (v/v) methanol and 10% (v/v) acetic acid. Subsequently, the purified protein band was electroblotted onto a Millipore polyvinylidene fluoride membrane according to Cheng (1998) for *N*-terminal sequencing. Protein bands identified by Coomassie Blue were excised and subjected to Edman degradation using a Procise 491 Protein Sequencer (America) in Chinese academy of medical sciences (Beijing, China). Analysis of the β-1,3-glucanase amino acid sequence with other proteins was done using the BlastX (http://www.ncbi.nlm.nih.gov/BLAST/).

Effects of β-1,3-glucanase on mycelia growth and spore germination

To determine the effects of β-1,3-glucanase on mycelia growth of *R. solani*, a 0.5 mm^2 agar disc

of a *R. solani* isolate was cut from a 10-day-old culture and mixed with 0.2 ml purified enzyme and incubated at 27°C for 12 h. Subsequently, the *R. solani* disc was transferred to water agar plate, incubated for 24 h at 27°C and the growth of the mycelium was determined. Heat-inactivated enzyme served as negative control.

About 200–300 spores of *Fusarium* sp. and *F. fulva*, respectively, were mixed with 0.2 ml purified β-1,3-glucanase and incubated at 27°C for 24 h. The suspension was placed under a light microscope to calculate the percentage of germinated spores. Incubation of spores with heat-inactivated β-1,3-glucanase served as control.

Results

Dual-culture plate analysis

As shown in Fig. 1, the hypha of *P. byssoides* and *R. solani* interacted with each other with the hypha of *P. byssoides* coiling around the hypha of *R. solani* (Fig. 1).

β-1,3-Glucanase production and purification

Periconia byssoides was cultivated in CM medium at 27°C with shaking at 200 rpm. The β-1,3-glucanase was detected on the second day and the enzyme activity reached the highest activity on the third day (Fig. 2). Purification factors and protein yield at each step of the purification procedure are summarized in Table 1. Analysis of the β-1,3-glucanase by SDS-PAGE revealed a band corresponding to a protein with a molecular mass of 35 kDa (Fig. 3).

Effects of temperature and pH on enzyme activity

The optimum reaction temperature of β-1,3-glucanase was at 50°C and the enzyme was stable below 30°C (Fig. 4A). The optimum pH for β-1,3-glucanase was pH 6.0 (Fig. 4B).

N-Terminal amino acid sequence

The ten N-terminal amino acid residues of the β-1,3-glucanase purified from *P. byssoides* are LKNGGPSFGA, which do not show any significant homology with other fungal β-1,3-glucanases (Table 2).

Effects of β-1,3-glucanase on mycelia growth and spore germination

The inhibitions between β-1,3-glucanase and plant pathogenic fungi *R. solani*, *Fusarium* sp. and *F. fulva* were studied according to the method described in

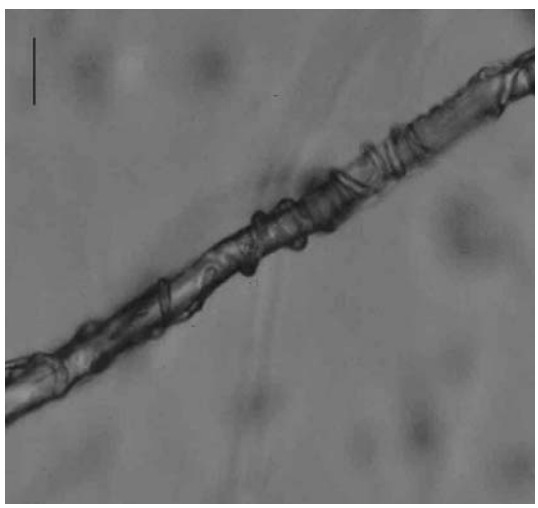

Fig. 1 *Periconia byssoides* coiled around the hypha of *Rhizoctonia solani*. The thick hypha is *R. solani*, and the slim hypha is *P. byssoides*. Magnification bars = 10 μm

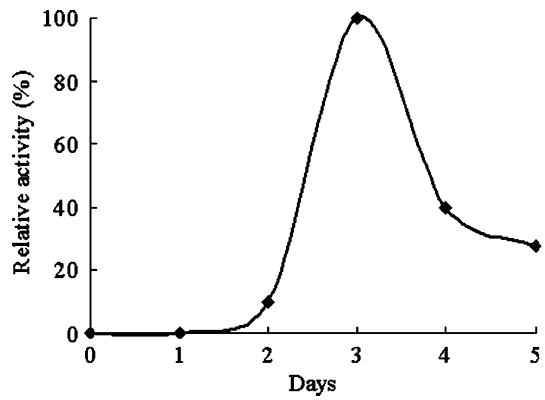

Fig. 2 Extracellular β-1,3-glucanase production over time in a CM broth liquid culture of *P. byssoides*. Activity of filtrate enzyme was measured using laminarin as substrate. The activity of β-1,3-glucanase at 3 days was defined as 100%, which corresponds to 3 U

Table 1 Purification of the β-1,3-glucanase from *Periconia byssoides*

Purification procedures[a]	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U mg^{-1})	Purification factor (fold)	Yield (%)
$(NH_4)_2SO_4$ precipitation	10	325	33	9.8	1	100
Hiprep 16/10 phenyl FF	15	103	5.1	20	2	32
HiTrap Q XL	3	88	2	44	4.5	27

[a] Cultures filtrate (1 l) were filtered under vacuum, and then the filtrate protein was precipitated with $(NH_4)_2SO_4$ (90% saturation) and collected by centrifugation (10,000*g*, 15 min). The resuspended precipitate was applied to a Hiprep 16/10 phenyl FF column (Amersham, Sweden) equilibrated with 50 mM NaH_2PO_4/NaOH buffer (pH 6.5) containing 1 M $(NH_4)_2SO_4$. Bound proteins were eluted with 50 mM NaH_2PO_4/NaOH buffer (pH 6.5). Fractions containing β-1,3-glucanase activity were pooled and dialyzed in 10 mM Tris/HCl buffer (pH 8.0). The dialyzed sample was applied to a HiTrap Q XL column (Amersham, Sweden) equilibrated with 10 mM Tris/HCl buffer (pH 8.0), then eluted with 10 mM Tris/HCl buffer containing 1 M NaCl, pH 8.0. Protein fractions with β-1,3-glucanase activity were pooled and concentrated for determination of purity and properties

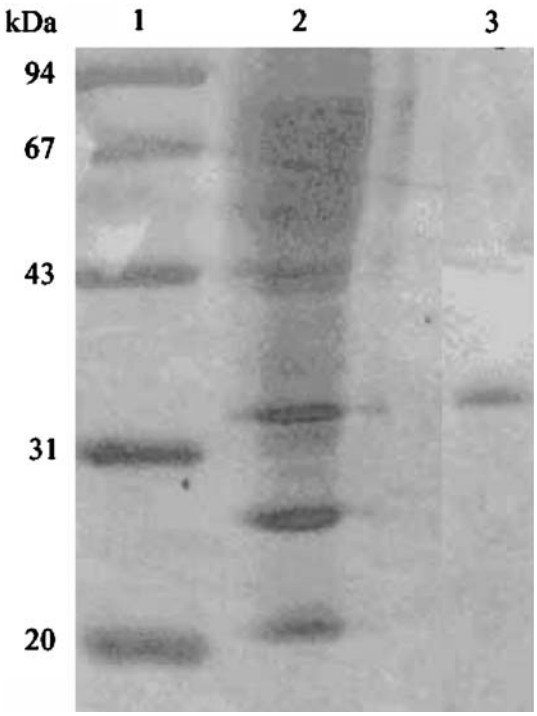

Fig. 3 SDS-PAGE analysis of the purified β-1,3-glucanase. Lane 1, Molecular mass marker; lane 2, crude extract; lane 3, purified β-1,3-glucanase

''Materials and methods''. The purified β-1,3-glucanase could inhibit mycelia growth and spore germination of plant pathogenic fungi: 60% growth of mycelia of *R. solani* was inhibited and more than 70% of *Fusarium* sp. and *F. fulva* spores could not germinate. Moreover, the purified β-1,3-glucanase degraded the cell walls of *R. solani*, *Fusarium* sp. and *F. fulva* (Table 3). The cell wall lytic activity of the β-1,3-glucanase was very strong in the presence of cell walls preparations from *R. solani* while β-1,3-glucanase showed lower activity to the cell walls preparations of *Fusarium* sp. and *F. fulva* (Table 3).

Discussion

β-1,3-Glucanase is one of the most important cell wall lytic enzymes and has been purified from a number of fungal species where the enzyme differs in physiological properties, molecular structure, and molecular weight (McQuilken and Gemmell 2004). The anti-fungal activity of β-1,3-glucanase varies between different plant pathogenic fungi and it was suggested that the activity of β-1,3-glucanase depends on the presence of β-1,3-glucan in the cell walls of the target organisms. It was also speculated that the activity of the enzyme may vary in response to the glucan structure and the type of linkage (Vazquez-Garciduenas et al. 1998).

The fungus *Periconia* is a soil inhabitant but may also be found in blackened and dead herbaceous steams and on leaf spots, grasses, rushes and sedges. Some species of *Periconia* can cause root-rot diseases in sorghum cultivars and as such *P. circinata* is considered a plant pathogenic fungus (Macko et al. 1992). Here, we report that *P. byssoides* may possibly protect plants by parasitizing plant pathogenic fungi as it has the

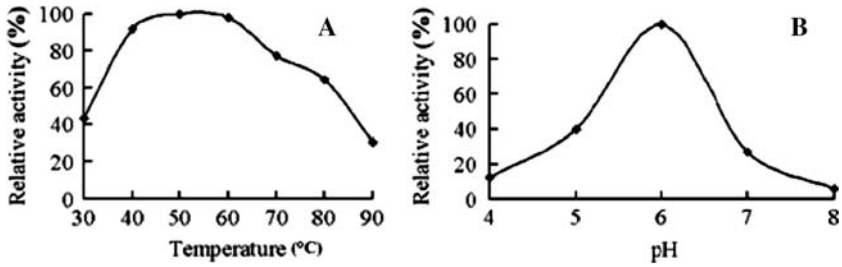

Fig. 4 (**A**) Effect of temperature on the activity of the purified β-1,3-glucanase. The optimum temperature was determined by incubating the reaction mixture at different temperatures ranging from 30 to 90°C. Residual activity was assayed according to the method described above. The activity of β-1,3-glucanase at 50°C was defined as 100%, which corresponds to 43.7 U. (**B**) Effect of pH on the activity of purified β-1,3-glucanase. The optimum pH was determined by incubating the reaction mixture in the barbital/NaOH buffer system (Yang et al. 2005) at different pH values ranging from 4 to 8 for 2 h. The activity of β-1,3-glucanase at pH 6 was defined as 100%, which corresponds to 43.7 U

Table 2 The N-terminal amino acid sequences of β-1,3-glucanases from different fungi

Source	Sequence	Reference
Periconia byssoides	LKNGGPSFGA	This work
Chaetomium sp.	_ _ P Y Q L Q T P _	Sun et al. (2006)
Lentinule edodes	L G T S A A A P L G	Minato et al. (2004)
Trichoderma harzianum	A T S F Y Y F N M D H V	Cruz et al. (1995)
Copernicia cerifer	S _ _ I A VY W E K	Cruz et al. (2002)

Table 3 Lytic activity of β-1,3-glucanase purified from *Periconia byssoides* towards cell walls preparations of three plant pathogenic fungi

Cell wall[a]	β-1,3-Glucanase (U)
Rhizoctonia solani	8.6
Fusarium sp.	0.14
Fulvia fulva	2.8

[a] Preparations of fungal cell walls were carried out according to the method described by Cruz et al. (1995). About 0.2 ml of purified β-1,3-glucanase was mixed with 2 mg cell walls from *R. solani*, *Fusarium* sp. and *F. fulva*, respectively. Mixtures were incubated at 40°C for 16 h with occasional shaking. The reactions were stopped by centrifugation (5,000*g*, 10 min) and the amount of reducing saccharides released in the supernatants was determined according to the method described by Miller (1959)

ability to produce extracellular enzymes that degrade the cell walls of other plant pathogenic fungi. Moreover, the purified N-terminal amino acid residues of β-1,3-glucanase secreted by *P. byssoides* do not share homology to β-1,3-glucanases purified from other species, suggesting it may be a novel member of fungal β-1,3-glucanases. In fact, alignments with available β-1,3-glucanase sequences from different fungi shows that they shared low conservative each other (Table 2), which suggests that the β-1,3-glucanases purified from different fungi share low homology, and β-1,3-glucanases secreted by *P. byssoides* is a novel protein with novel properties different from β-1,3-glucanase purified from other organisms (Cruz et al. 1995). We conclude that our work provides a sound basis for continued study of the molecular mechanisms of mycoparasitism in *P. byssoides* as this organism appears to be useful as a potential biocontrol agent for plant pathogenic fungi.

Acknowledgements We are grateful to Drs. Lemin Zhang, Linqian Dong and Ms. Wei Zhou for their helps, and Dr. Lei Cai for his advice to identify *P. byssoides*. The work was funded by the Department of Science and Technology of Yunnan Province (approved Nos. 20040001Z and 2005NG05).

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