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Purification and properties of a β-1,3-glucanase from *Chaetomium* sp. that is involved in mycoparasitism

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Abstract

A β-1,3-glucanase was detected, using laminarin as substrate, in the culture broth of *Chaetomium* sp. Major activity was associated with a 70 kDa protein band visualized on a polyacrylamide gel. β-1,3-Glucanase was purified by a one-step, native gel purification procedure. Optimal activity was observed at pH 6.0 and 30 °C (over 30 min). It could degrade cell walls of plant pathogens including *Rhizoctonia solani*, *Gibberella zeae*, *Fusarium* sp., *Colletotrichum gloeosporioides* and *Phoma* sp. The *N*-terminal amino acid residues of the purified β-1,3-glucanase are PYQLQTP, which do not exhibit homology to other fungal β-1,3-glucanases suggesting it may be a novel enzyme.

Introduction

Fungal biocontrol agents inhibit plant pathogens through one or more of the following mechanisms: mycoparasitism, competition for key nutrients and colonization sites, production of antibiotics, or stimulation of plant defense mechanisms (Vázquez-Garcidueñas *et al.* 1998). Among these, mycoparasitism relies on various lytic enzymes for the degradation of the cell walls of the hosts and these enzymes play an important role in infection of the host, such as chitinase and β-1,3-glucanase (Tweddell *et al.* 1994). The fungal cell wall protects the organism against a hostile environment and relays signals for invasion and infection of hosts. Fungi have a significant internal turgor pressure so that even slight perturbation of the cell wall can result in cell lysis (Selitrennikoff 2001). When the mycoparasite reaches its host, its hyphae will accrete and penetrate into the host's mycelia after partial degradation of the cell wall. β-1,3-Glucanase produced by antagonists is an important factor in this process. Some β-1,3-glucanases have been purified and the corresponding genes cloned (Takehara *et al.* 1981, Palumbo *et al.* 2003, Kozhemyako *et al.* 2004). However, the molecular background of how the mycoparasite infect the host has not been fully elucidated.

Members of the *Chaetomium* genus occur widely in nature, and certain *Chaetomium* spp. can produce biologically active metabolites, such as chaetochalasin A and chaetoglobosins, which suppress the growth of plant pathogens (Oh *et al.* 1998). Little research, however, has been carried out on the extracellular enzymes involved in its mycoparasitism. In this report, we described the purification, biochemical characterization, antifungal activity and *N*-terminal amino acid analysis of an extracellular β-1,3-glucanase from *Chaetomium* sp.

Materials and methods

Organisms and growth conditions

Chaetomium sp. and *Rhizoctonia solani, Gibberella zeae*, *Fusarium* sp., *Colletotrichum gloeosporioides*,

Phoma sp. and *Alternaria alternata* were obtained from the culture collection of the Laboratory for Conservation and Utilization of Bio-resources, Yunnan University, China. *Chaetomium* sp. was grown on potato/dextrose/agar (PDA) and then on a medium containing (g l^{-1}): K_2HPO_4 1, $MgSO_4 \cdot 7H_2O$ 0.25, NaCl 0.1, $FeSO_4 \cdot 7H_2O$ 0.005, $MnSO_4$ 0.005, $ZnSO_4$ 0.005, yeast extract 1, peptone 1 and oatmeal 1. The pH of the medium was adjusted to 6.5. Flasks (250 ml) containing 100 ml medium were shaken at 200 rpm at 30 °C for 6 d.

Preparation of fungal cell walls

Preparation of fungal cell walls used for determination of lytic activity of β-1,3-glucanase (see Table 2) was done according to the method of Tweddell *et al*. (1994). *Rhizoctonia solani*, *G. zeae*, *Fusarium* sp., *Coll. gloeosporioides* and *Phoma* sp. were scraped from the PDA after 6 d. These mycelia were freeze-dried, ground to a powder in liquid N_2, suspended in distilled water (0.2 g ml^{-1}), and centrifuged at 10 000 *g* for 10 min. This extraction procedure was repeated several times until no protein and glucose could be detected in the supernatant. After the final centrifugation step, the deposit, a homogeneous, gel-like substance, was gently scraped off to dry and stored at room temperature.

β-1,3-Glucanase activity assay

The enzyme solution (100 μl) was mixed with 200 μl 0.2% (w/v) laminarin (Sigma, USA) dissolved in 0.1 M buffer NaH_2PO_4/Na_2HPO_4 (pH 6.0) and incubated at 30 °C for 30 min. The reaction was terminated by adding dinitrosalicylic acid solution and boiling the reaction mixture for 5 min. The absorbance at 540 nm was measured. One unit (U) of β-1,3-glucanase activity was defined as the amount of the enzyme that released reducing sugar equivalent to 1 μg glucose per min under the above conditions.

Purification of β-1,3-glucanase

Unless otherwise indicated, the following steps were performed at 0–4 °C. The culture filtrate was fractionized using $(NH_4)_2SO_4$ and the fraction that precipitated between 55% and 85% saturation was collected. The final precipitate was resuspended in a minimal amount of buffer (pH 6.0), dialyzed against 10 mM sodium phosphate buffer (pH 6.0) overnight and stored at 4 °C until further purification.

The purification of β-1,3-glucanase was conducted as described by Wang *et al*. (2001) with some modifications. The crude enzyme solution was fractionated on a 15% native gel electrophoresis after being concentrated with PEG-6000. After electrophoresis, a strip of the gel was stained with Coomassie Brilliant Blue R-250 to localize protein bands. The protein bands from the unstained gel strip were excised separately. Part of the band was used to assay the enzyme activity, and the residual gel was electroeluted. The eluent was dialyzed against 10 mM sodium phosphate buffer (pH 6.0) overnight and stored at −20 °C until needed.

Effect of temperature and pH on enzyme activity

The optimum temperature was determined by carrying out the reaction from 20 to 80 °C. Enzyme stability was determined at various temperatures over 30 min with residual activity being measured as described above.

Optimum pH was also carried out at different pH values at 30 °C and pH stability of the enzyme was studied by incubating the samples at 30 °C at various pH values (10 mM sodium acetate buffer) for 30 min with the residual activity measured as described above.

Effect of metal ions on purified enzyme activity

The effects of metal ions were assayed by incubating the enzyme with its substrate (laminarin) adding different metal ions (5 mM) at 30 °C and pH 6.0 for 30 min.

Results

Purification of β-1,3-glucanase

Chaetomium sp. produced β-1,3-glucanase throughout growth (Figure 1), with maximum activity after 6 d.

The purified β-1,3-glucanase showed a single protein band upon SDS-PAGE. Its molecular mass was estimated to be 70 kDa (Figure 2)

which is similar to that of β-1,3-glucanases from other microorganisms (Miyanishi *et al.* 2003, Sanz *et al.* 2005).

Properties of purified β-1,3-glucanase

Optimum temperature for the activity of the β-1,3-glucanase was 30 °C at pH 6.0 for 30 min. It was stable below 30 °C but inactivated above 60 °C (over 30 min). The β-1,3-glucanase exhibited maximum activity at pH 6.0. The enzyme retained 50% of its optimal activity at pH 5.0 and 7.0.

β-1,3-Glucanase was activated by about 80% with 5 mM Mn^{2+} but 5 mM Fe^{3+} (pH 6.0) inhibited activity by 50%. However, Ca^{2+}, Cu^{2+}, Mg^{2+} and Zn^{2+} had little effect on activity.

Antifungal activity analysis

Inhibition to the germination of spores

After 24 h an almost complete inhibition in the germination of *Fusarium* sp. conidia could be seen, whereas germination in the control test was normal. The purified β-1,3-glucanase failed to inhibit the germination of A. *alternata* conidia (Table 1).

Assay for fungal cell wall lytic activity

The purified β-1,3-glucanase was able to hydrolyze cell walls of various plant pathogens. The enzyme alone was quite active against cell walls from *Phoma* sp., *R. solani* and *G. zeae*, but showed less activity against cell walls from *Fusarium* sp. and *Coll. gloeosporioides* (Table 2).

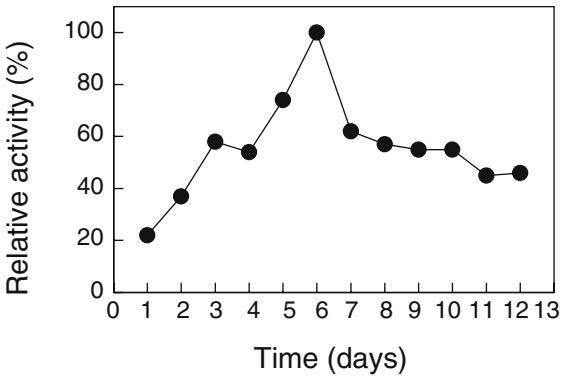

Fig. 1. Time course for β-1,3-glucanase production by *Chaetomium* sp. The enzyme activity of the culture fluid was monitored at 24 h intervals for 12 d. All values represent the means of three replicates. The activity of β-1,3-glucanase at the 6 d was defined as 100% which corresponds to 110 U ml^{-1}.

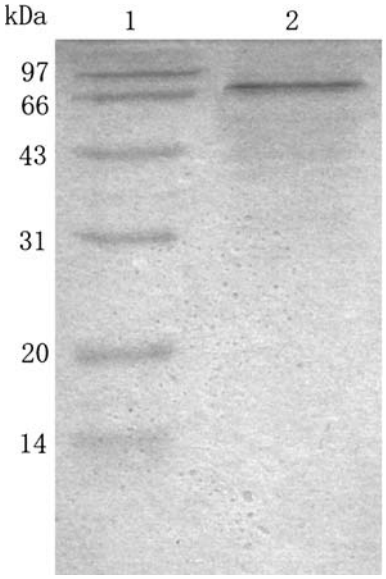

Fig. 2. SDS-PAGE (12%) was performed according to the method of Laemmli (1970) under reducing conditions. SDS-PAGE was used to analyze the molecular weight of the purified β-1,3-glucanase. Lane 1, molecular weight markers; lane 2, purified β-1,3-glucanase.

Interaction between Chaetomium *sp. and* R. solani

The interaction between *Chaetomium* sp. and *R. solani* is shown in Figure 3. When grown together, the *Chaetomium* sp. hyphae coiled

Table 1. The effect of purified β-1,3-glucanase on the spore germination of *Fusarium* sp. and *A. alternata.*

Fungus	Germination rate[a]	
	β-1,3-Glucanase (%)	Heat-inactivated β-1,3-glucanase (%)
Fusarium sp.	0	55
Alternaria alternata	29	29

Spores (1.0×10^6) of *Fusarium* sp. and *A. alternata* were incubated with purified enzyme solution (0.2 ml) at 27 °C for 24 h (retained 80% of primary activity). Then the suspensions were microscopically examined. The percentage of germinated spores was determined as the percentage of the first 100 spores randomly found in the visual field and with the heat-inactivated (100 °C, 20 min) enzyme as control.

[a]Germination rate: germinated spores/tested spores.

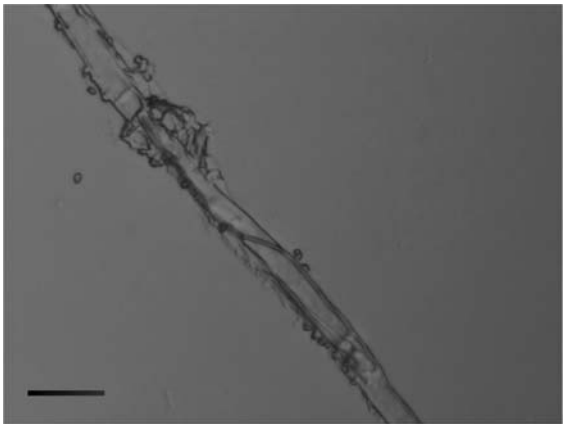

Fig. 3. Chaetomium sp. and *R. solani* were grown opposite each other on a water/agar medium (2% agar) for 15 d, and the interaction between *Chaetomium* sp. and *R. solani* observed under a light microscope. The hyphae of *Chaetomium* sp. coiled around *R. solani* (the hyphae of *R. solani* is thick, and the hyphae of *Chaetomium* sp. is very thin). Scale bar = 20 μm.

Table 2. Relative activity of β-1,3-glucanase purified from *Chaetomium* sp. towards various preparations of fungal cell walls.

Substrate (cell wall)	Relative activity (%)[a]
Laminarin	100
Gibberella zeae	11
Fusarium sp.	6
Colletotrichum gloeosporioides	8
Phoma sp.	17
Rhizoctonia solani	14

The purified enzyme (400 μl) was added to the cell wall suspension (0.2 g ml^{-1}, 400 μl). The mixture was incubated at 37 °C for 8 h (retained 60% of primary activity) and then centrifuged at 10 000 *g* for 5 min. The amount of glucose solubilized in the supernatant was determined by the phenol/sulfuric acid reagent.

[a]Relative activity is expressed as a percentage of that toward laminarin, 100% activity corresponds to 49 U ml^{-1}.

around the *R. solani* hyphae, and the *R. solani* hyphae was degraded partially.

N-Terminal amino acid sequence analysis

The residues of the *N*-terminal amino acid of the purified enzyme were determined and the results obtained were shown in Table 3. No homology to other fungal β-1,3-glucanases was found.

Discussion

β-1,3-Glucanases have been purified from many microorganisms. There is increasing evidence that they play important roles in mycoparasitism (Vázquez-Garcidueñas *et al.* 1998, Sanz *et al.* 2005). In this study, we isolated an extracellular β-1,3-glucanase from the culture of *Chaetomium* sp. and its *N*-terminal amino acids had no homology with other β-1,3-glucanases. This enzyme could inhibit the spore germination of plant pathogens *Fusarium* sp. and degrade the cell walls of *Phoma* sp., *Fusarium* sp., *Coll. gloeosporioides, R. solani* and *G. zeae*. But the interaction between host and mycoparasite is very complicated. Further studies are necessary to ascertain the role of the β-1,3-glucanase and the cooperation with other factors in mycoparasitism.

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

Source	Sequence	Reference
Chaetomium sp.	_ _ P Y Q L Q T P _	This work
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 cerifera	S _ _ I A VY W E K	Cruz *et al.* (2002)

The proteins were separated on a 12% SDS-PAGE gel and electroblotted to a PVDF membrane for *N*-terminal sequencing. *N*-Terminal amino acid sequencing was performed on a PE/ABI PROCISE491 Protein Sequencer.

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