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Partial purification and characterization of a 1,3-β-D-glucanase from *Ganoderma tsugae*

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SUMMARY

The crude broth from a submerged culture of *Ganoderma tsugae* was assayed for activity against laminarin, and enzyme staining indicated the presence of 1,3-β-D-glucanases. 1,3-β-D-glucanase activity increased rapidly after the stationary growth phase of *Ganoderma tsugae*. At the same time the glucose was almost depleted and the amount of extracellular polysaccharide decreased in the broth medium. There were two 1,3-β-D-glucanase isozymes in the culture broth of *Ganoderma tsugae*. After gel filtration, the first peak showed a high activity for laminarin; it was designated as 1,3-β-D-glucanase (I). The second peak exhibited much less activity; it was designated as 1,3-β-D-glucanase (II). Since 1,3-β-D-glucanase (II) activity was very low, and there were probably some inhibitors present in the 1,3-β-D-glucanase (II) fractions, we collected only the fractions that contained 1,3-β-D-glucanase (I) activity. 1,3-β-D-glucanase (I) was specific for laminarin and was found to hydrolyze extracellular polysaccharide in the culture broth of *Ganoderma tsugae*. The maximum activity of 1,3-β-D-glucanase (I) was at about 50 °C and pH 5.0. The enzyme was stable at pH 5.0 and at temperatures below 40 °C.

INTRODUCTION

The antitumour activity of polysaccharide preparations from fungi, such as *Auricularia auricula-judae* [12], and *Pestalotia* sp. 815 [13] has been reported. 1,3-β-D-glucan, a polysaccharide preparation from the fruiting body and the mycelium of *Ganoderma lucidum* also has antitumour activity [19], and has attracted attention to the polysaccharides of *Ganoderma* species. However, *Ganoderma* species are phytopathogenic fungi [2,21]. In order to protect woody plants from *Ganoderma* species, a submerged culture of *Ganoderma* species was chosen to produce extracellular polysaccharides. During the course of extracellular polysaccharide production from *Ganoderma tsugae* grown in culture, it was noted that extracellular polysaccharide underwent slow hydrolysis at the stationary phase [8]. Hydrolysis was considered to be due to an associated 1,3-β-D-glucanase [1,17]. In this study, we report some properties of 1,3-β-D-glucanase from *Ganoderma tsugae* and the association between 1,3-β-D-glucanase and the extracellular polysaccharide produced in culture by this fungus.

MATERIALS AND METHODS

Culture conditions and growth measurement

Ganoderma tsugae 1109 from our culture collection was precultured on potato dextrose agar (Difco, Detroit, MI, USA) at 28 °C for 7 days. Mycelium was harvested after 7 days. Mycelium fragments from three plates were transferred into 100 ml of sterilized water and homogenized with a blender. Liquid cultures were grown using 5 ml of this suspension as inoculum into 500-ml flasks containing 200 ml of medium (30 g L^{-1} glucose, 2 g L^{-1} Bacto-peptone, 6 g L^{-1} yeast extract) and shaking the flasks (120 r.p.m.) at 28 °C on an orbital shaker.

Growth was determined by monitoring the mycelial dry weight every 3 days for 4 weeks. Mycelium was separated from culture broth by centrifugation and the supernatant was used for enzyme assay and purification. The pellet was dried to a constant weight under reduced pressure at 40 °C.

Determination of residual glucose and extracellular polysaccharide concentration during growth

Glucose was measured with a peroxidase/glucose oxidase kit (Sigma, St Louis, MO, USA). Extracellular polysaccharide was determined by the phenol-sulfuric acid method [3].

Purification of the enzyme

Step one: salt precipitation. The supernatant was subjected to ammonium sulfate precipitation at 4 °C. The precipitate that formed between 20% and 70% saturation of ammonium sulfate was collected by centrifugation at 10 000 × *g*. The precipitate was dialyzed overnight against sodium acetate buffer (10 mM, pH 5.0).

Step two: gel fitration. The redissolved ammonium sulfate precipitate was applied to a column of Sephadex G–75 (16 × 900 mm) equilibrated with 10 mM sodium acetate, pH 5.0. The column was eluted with the same buffer at a rate of 0.35 ml min^{-1}. Fractions (3.5 ml each) were collected and analyzed for 1,3-β-D-glucanase activity, and protein concentration was determined by A_{280}. Those fractions showing 1,3-β-D-glucanase activity were pooled and stored at 4 °C.

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Enzyme staining

The redissolved ammonium sulfate precipitate was loaded onto native polyacrylamide gel electrophoresis (PAGE) gel. PAGE was carried out on 12.5% polyacrylamide gel (9 × 8 cm) at pH 8.3 using 100 V for 120 min [5]. After electrophoresis, the gel was washed with distilled water three times [16], and incubated in 0.05 M potassium acetate (pH 5.0) for 5 min with slow shaking. The gel was then incubated at 40 °C for 30 min in a mixture containing 75 ml of the same buffer and 0.5 g laminarin dissolved in 75 ml water by heating in a boiling water bath. The gel was incubated in a mixture of methanol, water, acetic acid (5:5:2) for 5 min, washed three times with distilled water after incubation and then put into a glass tray containing 0.3 g 2,3,5-triphenyltetrazolium chloride in 200 ml 1.0 M NaOH. The tray was kept in a boiling water bath until red bands appeared. The gel was then stained for proteins with Coomassie Brilliant Blue R.

Enzyme assay

The assay of 1,3-β-D-glucanase was based on release of reducing sugars from laminarin. 1,3-β-D-glucanase activity was assayed by incubating 1 ml laminarin (5 g L^{-1}) in 10 mM sodium acetate buffer, pH 5.0 with 1 ml of enzyme solution at 50 °C for 10 min. The reaction was terminated by immersing the samples in a water bath (100 °C) for 10 min. Two milliliters of 3,5-dinitrosalicylic acid (DNS) reagent were then added and the mixture was heated in a 100 °C water bath for 15 min. As a control, 1 ml of laminarin solution with 1 ml of enzyme solution was heated in a 100 °C water bath for 10 min and 2 ml of DNS reagent were added to correct for the reducing sugars in the substrate and the enzyme solution. Reducing sugar equivalents were measured in both the original and the

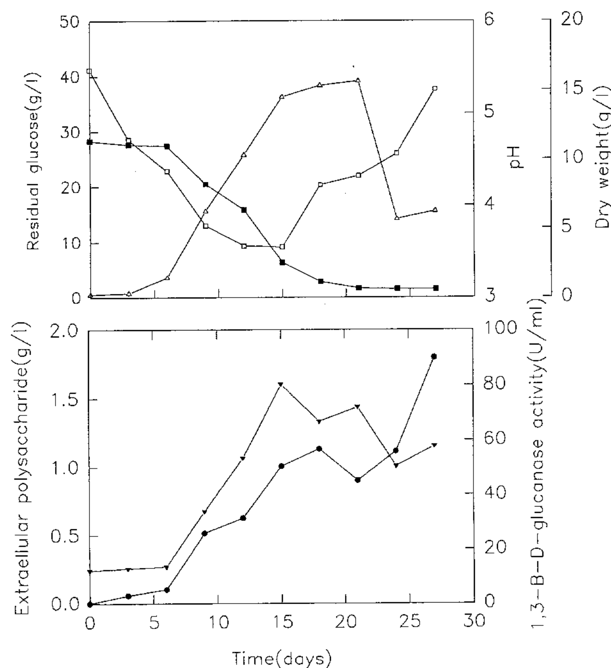

Fig. 1. Change of dry weight (△—△), pH (□—□), residual glucose (■—■), extracellular polysaccharide (▼—▼), and 1,3-β-D-glucanase activities (●—●) during the cultivation of *Ganoderma tsugae*.

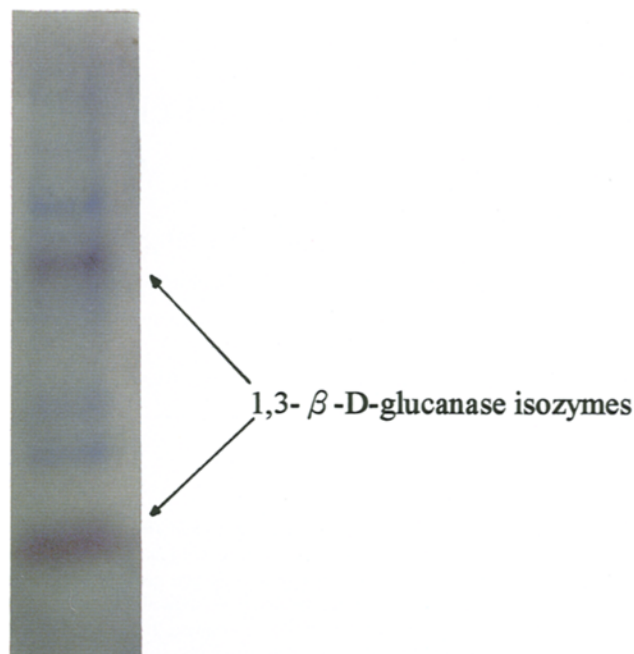

Fig. 2. Two 1,3-β-D-glucanase isozymes found in the culture broth of *Ganoderma tsugae*. The arrows indicate 1,3-β-D-glucanase isozyme bands, which appear red after staining with Coomassie Brilliant Blue R, whereas the other proteins were visualized as blue bands.

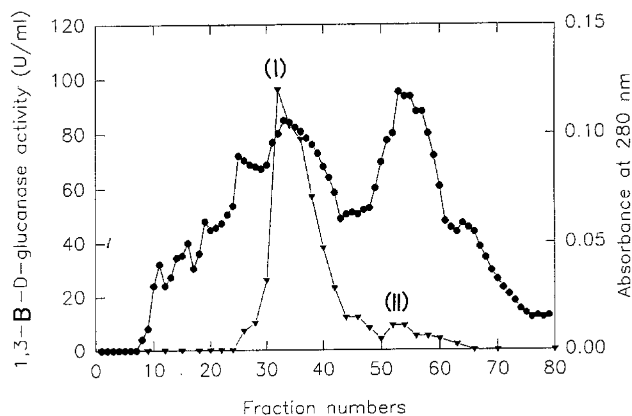

Fig. 3. Gel filtration of ammonium sulfate precipitated 1,3-β-D-glucanases on Sephadex G-75. Fractions were assayed for protein (●—●) and 1,3-β-D-glucanase activity (▼—▼).

control solutions by the colorimetric method of Miller et al. [11] with glucose as the standard. One unit of 1,3-β-D-glucanase activity was defined as the enzyme activity that released 1 nmol of reducing sugars (determined as glucose) per min under the assay conditions. Protein concentration was estimated by the method of Lowry et al. [9] with bovine serum albumin as standard.

Effect of temperature on activity and stability of the enzyme

The enzyme activity was determined at various temperatures to determine optimum temperature for the activity. For the effect of temperature on stability, the enzyme was incubated at various temperatures for 60 min in 10 mM acetate

buffer (pH 5.0). After incubation, the residual activity was assayed under the conditions described above.

Effect of pH on activity and stability of the enzyme

The enzyme activity was measured at various pH values from 3.5 to 6.0 (acetate buffer). For the effect of pH on stability, the enzyme was preincubated at 25 °C for 60 min at various pH values and the residual activity was determined under the conditions described above.

RESULTS AND DISCUSSION

Time course of enzyme production

Figure 1 shows the substrate consumption, pH, extracellular polysaccharide, dry weight of mycelium, and 1,3-β-D-glucanase at different stages of growth. From day 15 to 21, the growth of the organism reached its maximum. After 21 days cultivation, when the concentration of glucose had decreased to about 1 g L^{-1}, the mycelium underwent autolysis and the amount of extracellular polysaccharide decreased, the enzyme activity increased rapidly. These results were similar to those of Rapp [18]. The enzyme activity increased during continued starvation while mycelium and extracellular polysaccharide were degraded.

Enzyme staining after PAGE and purification of the enzyme

The crude enzyme was prepared from the culture broth harvested at the stationary phase of growth. The precipitate from 20–70% ammonium sulfate saturation of the culture broth was collected, and dissolved in 10 mM sodium acetate (pH 5.0). The dissolved enzymes were loaded on the polyacrylamide gel and ran as described above. The gel was then incubated with laminarin, and 1,3-β-D-glucanase isozymes were detected by using 2,3,5-triphenyltetrazolium chloride (data not shown). The gel was then stained with Coomassie Brilliant Blue R. The 1,3-β-D-glucanase bands appeared red, whereas the other proteins were visualized as blue bands (Fig. 2).

After gel filtration, two main peaks with laminarin-degrading activity were eluted (Fig. 3), but one peak, which was the first eluted, showed a high activity for laminarin, and was designated as 1,3-β-D-glucanase (I). The other peak, which was the second eluted, exhibited much less activity, and was designated as 1,3-β-D-glucanase (II). Since there were probably some inhibitors present in the 1,3-β-D-glucanase (II) fractions, we collected only the first peak fractions which contained 1,3-β-D-glucanase (I) activity. Table 1 summarizes the purification of 1,3-β-D-glucanase (I).

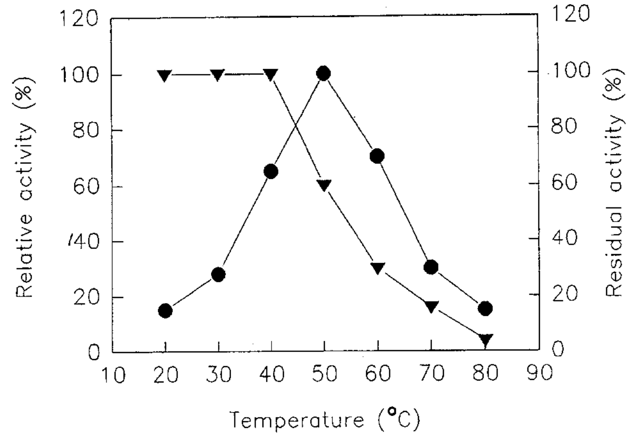

Fig. 4. Effect of temperature on 1,3–β-D-glucanase (I) activity (●—●) and stability (▼—▼).

Effect of temperature and pH on 1,3-β-D-glucanase (I)

As shown in Fig. 4, the maximum activity was obtained at about 50 °C, which was higher than that reported for the same enzyme from *Lentinus lepideus* [20]. The enzyme was sensitive to temperatures above 40 °C, and at 80 °C, the activity was almost completely lost.

Figure 5 shows the optimum pH at about 5.0 which was similar to that for enzyme from *Lentinus lepideus* [20] The enzyme was stable at pH 5.0. It was considerably unstable under these conditions at other pH values, and lost part of its activity. The pH stability of the enzyme was lower than that for the *Lentinus lepideus* system [20].

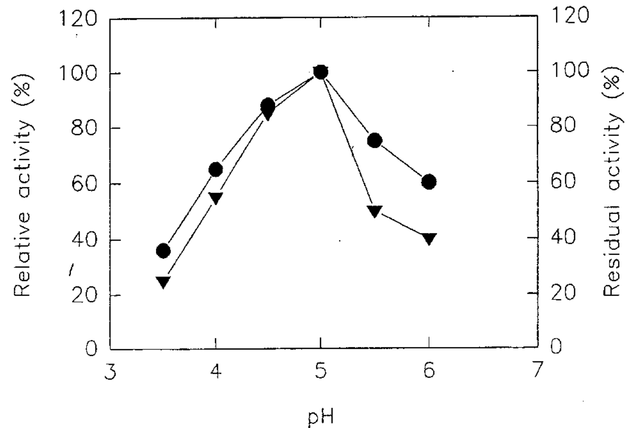

Fig. 5. Effect of pH on 1,3–β-D-glucanase (I) activity (●—●) and stability (▼—▼).

TABLE 1

Summary of purification of 1,3-β-glucanase (I)

Purification step	Total vol. (ml)	Total protein (mg)	Total activity (units)	Sp. activity (units mg^{-1})	Purification (fold)	Yield (%)
Culture filtrate	1700	2108	81 600	38.7	1.0	100
20–70% $(NH_4)_2$ SO_4	93	365	53 160	145.6	3.76	65.1
Sephadex G-75	62	28	16 316	582.7	15.05	20.0

TABLE 2

Substrate specificity of the purified 1,3-β-glucanase (I) from *Ganoderma tsugae*

Substrate	Monomer	Linkage type	Relative activity
Laminarin	glucose	1,3-β-	100%
Curdlan (bacteria)	glucose	1,3-β-	0%
Zymosan (yeast)	glucose	1,3-β- & 1,6-β-	0%
Lichenan	glucose	1,3-β- & 1,4-β-	70%
Xylan	xylose	1,4-β-	0%
Dextran	glucose	1,3-α- & 1,6-α	0%
Starch (potato)	glucose	1,4-α- & 1,6-α-	0%
Amylopectin	glucose	1,4-α- & 1,6-α-	0%
Cellulose	glucose	1,4-β-	0%

The assays were performed for 10 min at 50 °C. The assay mixture (1 ml) contained purified enzyme plus buffer. Substrate concentration was 2.5 mg ml^{-1} in 1 mM sodium acetate buffer, pH 5.0.

Substrate specificity of 1,3-β-D-glucanase (I)

In this experiment (Table 2), the activities on lichenan were 70% relative to that of laminarin. Under these conditions, curdlan, zymosan, xylan, dextran, starch, amylopectin, and cellulose were not attacked by the enzyme. 1,3-β-D-glucanase (I) seems to prefer 1,3-β-D-linkages, but is capable of hydrolyzing glucan containing both 1,3-β-D- and 1,4-β-D-glucosidic linkages.

Treatment of laminarin and extracellular polysaccharide with purified 1,3-β-D-glucanase (I)

Figure 6 shows the products at different times of the hydrolysis of laminarin with purified 1,3-β-D-glucanase (I). The laminarin substrate was hydrolyzed to reducing sugar and free glucose, but the increasing ratios of the reducing sugar product and free glucose product did not show a relationship. This suggests that the 1,3-β-D-glucanase (I) is an endo-type enzyme in its mode of action. Figure 7 shows the products at different times of the hydrolysis of extracellular polysaccharide (2.32 mg ml^{-1}) from a 24-day-old culture with purified 1,3-β-D-glucanase (I). The extracellular polysaccharide was hydrolyzed to a certain amount of reducing sugar and little free glucose. This suggests that the extracellular polysaccharide from the culture broth of *Ganoderma tsugae* underwent slow hydrolysis in stationary phase, and this hydrolysis was due to the action of 1,3-β-D-glucanase (I).

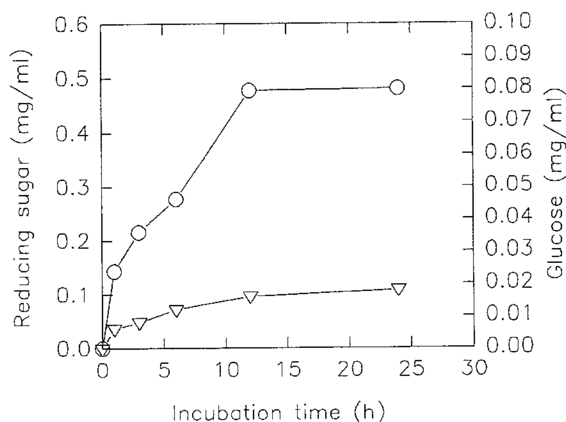

Fig. 6. Reducing sugar (O—O) and free glucose (▽—▽) production due to hydrolysis of laminarin by 1,3–β-D-glucanase (I).

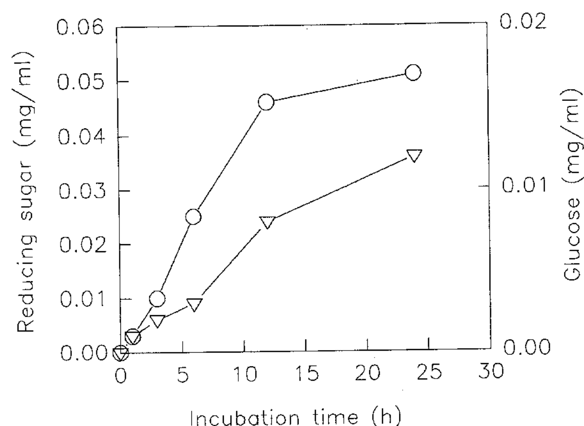

Fig. 7. Reducing sugar (O—O) and free glucose (▽—▽) production from the hydrolysis of extracellular polysaccharide by 1,3–β-D-glucanase (I).

Recently, attention has been directed to the antitumour activity of the extracellular polysaccharides from the culture broth of fungi [6,12,13,19,22]. The structures of extracellular polysaccharides were reported to contain a backbone chain of 1,3-linked D-glucose residues and branch chains of 1,6-linked D-glucose units [19]. However, a number of studies demonstrated that 1,3-β-D-glucanase is often found in the culture broth of fungi [4,7,10,14,15,17,18,20]. In this study, two 1,3-β-D-glucanase isozymes were detected in the culture broth of *Ganoderma tsugae* by using 2,3,5-triphenyltetrazolium chloride. We tried to isolate and partially purify 1,3-β-D-glucanases, but in the gel filtration we chose to collect only 1,3-β-D-glucanase (I), because of low activity and the probable presence of some inhibitors in 1,3-β-D-glucanase (II) fractions. In the treatment of extracellular polysaccharide with purified 1,3-β-D-glucanase (I), the results suggested that the decrease in yield

of extracellular polysaccharide during the stationary phase resulted from 1,3-β-D-glucanase (I) in the culture broth.

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