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Changes in (1 → 3)-β-Glucanase Activities during Stipe Elongation in *Coprinus cinereus*

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Abstract. Cell-free extracts and cell wall autolysates prepared from the stipes of basidiocarp of *Coprinus cinereus* were examined for (1 → 3)-β-glucanase activities. Gel filtration revealed two major peaks and a minor one of (1 → 3)-β-glucanases in both of the preparations, the former ones being designated as glucanase I and glucanase II. Glucanase I with a molecular weight of 300,000 had activity toward *p*-nitrophenyl-β-D-glucoside (pNPG) as well as laminarin, whereas glucanase II with a molecular weight of 70,000 had no activity toward pNPG. Both enzymes had only negligible activity toward pustulan. During stipe elongation, the level of glucanase-II activity remarkably increased with increasing rate of the elongation, whereas that of glucanase-I activity remained almost constant, in both the cell-free extract and the cell wall autolysate. Near the end of stipe elongation, both glucanase activities were lowered in the cell wall autolysate, but remained high in the cell-free extract.

Stipe elongation during basidiocarp maturation in *Coprinus cinereus* is a useful system for studying hyphal elongation, because the elongation process is due to the elongation of component cells, and its rate is remarkably high [4, 6]. During stipe elongation in *C. cinereus*, the mechanical properties of the stipe cell wall change [6], and the modifications of the component polysaccharides of the cell wall occur, which involve the decrease of molecular weight [7]. These suggest that wall-lytic enzymes are involved in stipe elongation. In fact, glycanase activities exist in the crude cell wall fraction of the stipe [8]. Furthermore, a positive relation has been found between the rates of stipe elongation and the autolysis in vitro of the stipe cell walls [9]. However, the lytic enzymes in the cell walls have not yet been fully characterized.

In the present investigation, (1 → 3)-β-glucanases in the stipes of *C. cinereus* at different stages of elongation were extracted and analyzed in order to understand the possible role of the cell wall hydrolysis in the mechanisms of stipe elongation.

Materials and Methods

Organism and culture conditions. The wild-type dikaryotic stock (no. 5026 + 5132, ATCC 56838) of *Coprinus cinereus* (Schaeff. ex Fr.) S.F. Gray *sensu* Konr. was used. An agar block with mycelium (2 × 2 mm) was inoculated on the center of CY-1 agar medium in Petri dishes 9 cm in diameter [9], and incubated at 28°C under a regime of 12 h light/12 h dark. Under these conditions, basidiocarp maturation occurred ten or 11 days after inoculation.

Fractionation of stipe cells. Stipes (5–10 g fresh wt) were homogenized in 50 ml McIlvaine buffer (pH 5.0) [10] containing 0.01% (wt/vol) sodium azide in a Waring blender at 15,000 rpm for 1 min and then broken with a French press (Ohtake model 5615, Tokyo, Japan) operated at 159 MPa. The homogenate was centrifuged at 1000 *g* for 5 min. The supernatant was further centrifuged at 10,000 *g* for 20 min, and its supernatant was used as cell-free extract. On the other hand, the pellet after the 1000 *g* centrifugation was purified for cell walls according to the method described by Santos et al. [12] with modifications as described by Kamada et al. [9]: the pellet was washed twice with McIlvaine buffer (pH 5.0) containing 0.01% (wt/vol) sodium azide and 0.75 *M* NaCl and then three times with the same buffer containing sodium azide only by low-speed centrifugations. All the above operations were carried out at 4°C.

Autolysis of cell walls. Cell walls purified as above were resuspended in 10 ml McIlvaine buffer (pH 5.0) containing 0.01% (wt/vol) sodium azide, and incubated with continuous shaking (50 strokes min^{-1}) at 37°C as described by Kamada et al. [9]. The enzymes liberated by autolysis were recovered in the supernatant after centrifugation at 2000 *g* for 10 min.

Gel filtration. Filtrations were performed on columns of Bio-gel P-200 (1.1 × 44 cm) and Bio-gel A-5m (1.1 × 45 cm) previously equilibrated with McIlvaine buffer (pH 5.0). The columns were

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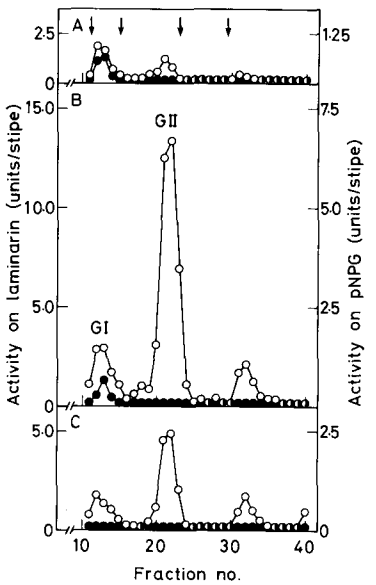

Fig. 1. Bio-gel P-200 gel filtration of enzyme preparations from the cell wall autolysates of the stipes of *Coprinus cinereus*. Samples prepared from the stipes at the 6-h (A), 15-h (B), and 21-h (C) stages were gel-filtrated, and the activities on laminarin (○) and *p*-nitrophenol-β-D-glucoside (pNPG) (●) in each fraction were assayed as described in *Materials and Methods*. The arrows indicate the elution positions of (left to right) thyroglobulin (mol. wt 670,000), gamma globulin (158,000), ovalbumin (44,000), and myoglobin (17,000). GI, glucanase I; and GII, glucanase II.

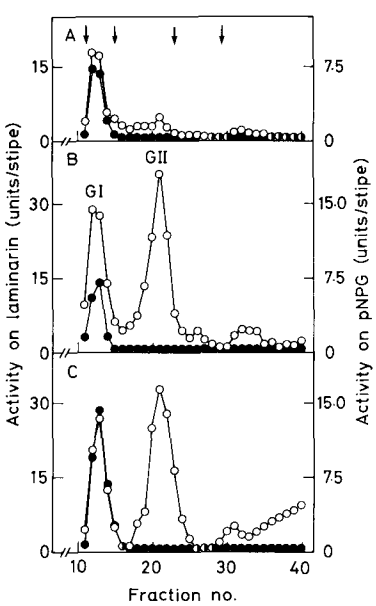

Fig. 2. Bio-gel P-200 gel filtration of cell-free extracts from the stipes of *Coprinus cinereus*. Samples prepared from the stipes at the 6-h (A), 15-h (B), and 21-h (C) stages were gel-filtrated, and the activities on laminarin (○) and *p*-nitorophenyl-β-D-glucoside (pNPG) (●) in each fraction were assayed as described in *Materials and Methods*. The arrows, GI, and GII are the same as for Fig. 1.

calibrated with Gel Filtration Standard containing thyroglobulin (mol. wt 670,000), gamma globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B-12 (1350). Samples (0.5 ml) were eluted at a rate of 1 ml h^{-1}, and fractions of 1 ml were collected.

Enzyme assays. Reaction mixtures contained 0.2 ml of enzyme preparation plus 0.2 ml of 0.2% (wt/vol) laminarin, 0.2% (wt/vol) pustulan, or 0.1% (wt/vol) *p*-nitrophenyl-β-D-glucoside (pNPG) in McIlvaine buffer (pH 5.0). On assays on the glucans, the mixtures were incubated at 37°C for 30 min, and the reactions stopped by boiling at 100°C for 5 min. On assays on pNPG, the mixtures were incubated at 37°C for 60 min, and the reactions stopped by adding 0.4 ml of 1 *M* Na_2CO_3. The activities on the glucans were estimated by measuring the released reducing sugar groups by the method described by Somogyi [14] in conjunction with that of Nelson [11] using glucose as standard. The activity on pNPG was determined by measuring the A_{410} of the *p*-nitrophenol released. One unit (U) of activity is defined as the amount of enzyme that liberated 1 nmol product per minute under reaction conditions.

Chemicals. Bio-gel P-200, Bio-gel A-5m, and Gel Filtration Standard were purchased from Bio-Rad Lab. Laminarin and pustulan were from Calbiochem-Behring. *p*-Nitrophenyl-β-D-glucoside (pNPG) and *p*-nitrophenol were from Sigma Chemical Company.

Results and Discussion

Solubilization of wall-associated glucanases. Under the culture conditions described above, stipe elongation during basidiocarp maturation proceeds in a synchronous manner [6]. The rate of elongation increases up to 18 h after the start of illumination on the day of basidiocarp maturation and then decreases rapidly [6]. Elongation ends by 24 h [6]. Cell walls freshly isolated from the stipes at 15 h after the start of the illumination (i.e., at a rapidly elongating stage) were incubated under autolytic conditions. (1 → 3)-β-Glucanase activity was rapidly released from the cell walls during the first 3 h of incubation, and thereafter the enzyme release became slow; about 50% of the bound enzyme was released during the first 3 h. The enzyme release during the 3-h autodigestion from the stipe cell walls at the 6-h stage (i.e., at the beginning of elongation) and that at the 21-h stage (i.e., near the end of elongation) were also about 50%.

Changes in (1 → 3)-β-glucanase activities during stipe elongation. Gel filtration on a Bio-gel P-200 column of the enzyme preparations from the cell wall autolysates after the 3-h autodigestion revealed two major peaks of (1 → 3)-β-glucanase activities, designated as glucanase I and glucanase II, and a minor peak of the activity (Fig. 1). Glucanase I, eluted near the void volume, showed activity to-

ward pNPG as well as toward laminarin (Fig. 1). The molecular weight of glucanase I was estimated to be 300,000 by gel filtration on a Bio-gel A-5m column. Glucanase II had a molecular weight of 70,000, and showed no activity toward pNPG (Fig. 1). Glucanases I and II were found to have only negligible activities toward pustulan by the examination of the peak fractions from the Bio-gel P-200 gel filtration. The level of the activity of glucanase II appeared lower than that of glucanase I in the stipe cell walls at the beginning of elongation (Fig. 1A). However, the glucanase-II activity increased remarkably during stipe elongation, whereas the increase of glucanase-I activity was slight (Fig. 1B). Both enzyme activities were fairly lowered near the end of elongation; especially, glucanase I appeared to lose the majority of the activity toward pNPG (Fig. 1C). The minor enzyme with a molecular weight of less than 17,000 had no activity toward pNPG like glucanase II, and its activity appeared to change in parallel with glucanase-II activity during stipe elongation (Fig. 1), suggesting some relation between these two enzymes. Further experimentation was, however, not made on this problem.

Gel filtration on a Bio-gel P-200 column of the cell-free extracts from the stipes revealed three peaks of (1 → 3)-β-glucanase activities corresponding to those found in the cell wall autolysates (Fig. 2). Although the ratio of glucanase I to glucanase II in the cell-free extracts was higher than that in the cell wall autolysates throughout the elongation process, the glucanase-II activity increased remarkably also in the cell-free extracts during stipe elongation (Figs. 1 and 2). The activities of glucanases I and II remained great near the end of stipe elongation (Fig. 2C).

Thus, the relative proportion of glucanase II to glucanase I was clearly increased in the stipe at a rapidly elongating stage, suggesting that glucanase II is important to stipe elongation. Endoglycanases that attack the glycosidic linkages at intermediate points of the polymer chain are postulated to play a crucial role in the fungal cell growth (1–3, 5, 13]. Glucanase II may be one of such endoglucanases because it has no activity toward pNPG (Figs. 1 and 2). To get further insight into the possible role of cell wall hydrolysis in the mechanisms of stipe elongation, however, glucanase II must be further characterized for the mode of action toward various polysaccharides, including those in the stipe cell wall.

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