Autolysis *in vitro* of the Stipe Cell Wall in *Coprinus macrorhizus*

By TAKASHI KAMADA,* YUZO HAMADA AND
TSUNEO TAKEMARU

Department of Biology, Faculty of Science, Okayama University, Okayama 700, Japan

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Cell walls isolated from rapidly elongating stipes of *Coprinus macrorhizus* basidiocarps lost 55% of their dry weight during 24 h incubation at 37 °C. Neutral sugars accounted for 73% of the solubilized products, glucose being the major sugar component. In the carbohydrate fraction solubilized after relatively short periods of autolysis, polymeric (degree of polymerization >10), dimeric and monomeric components were detected, and the larger components were converted to monosaccharide as the reaction proceeded. Three polysaccharide fractions of the cell wall were highly susceptible to autolytic enzymes, whereas another fraction was not. Chitin appeared to be relatively resistant. Analyses of the cell walls from various stages during stipe elongation revealed a positive relation between the rate of stipe elongation and the initial rate of autolysis, suggesting that the autolytic enzymes are involved in the mechanisms of stipe elongation.

INTRODUCTION

Stipe elongation during basidiocarp maturation in *Coprinus macrorhizus* is caused by the elongation of component cells, and the rate of elongation is conspicuously high (Kamada & Takemaru, 1977*a*). Thus, stipe elongation offers a useful system for clarifying the mechanism of hyphal cell elongation. The synthesis of chitin, a major component of the stipe cell wall, is required for stipe elongation (Gooday, 1973, 1975; Gooday *et al.*, 1976). On the other hand, it has been found that the mechanical properties of the stipe cell wall change in parallel with the elongation rate (Kamada & Takemaru, 1977*a*), and that some polysaccharide components of the cell wall decrease per unit stipe length as the stipe elongates (Kamada & Takemaru, 1977*b*). These results suggest that the degradation of cell-wall components is also involved in the elongation process. Recently, Kamada *et al.* (1980) detected (1→3)- β -glucanase, (1→6)- β -glucanase and chitinase activities in the crude cell-wall fraction of the stipe. However, there is no information about the activity of these wall-bound enzymes *in situ*, and their role in the elongation process is poorly understood. In the present study, the stipe cell walls of *C. macrorhizus* were autolysed *in vitro*, and the autolysing cell walls and their soluble products were analysed, in an attempt to obtain information concerning these problems.

METHODS

Organism and culture conditions. The wild-type dikaryotic stock (#5026+5132) of *Coprinus macrorhizus* Rea. f. *microsporus* Hongo was used. Moore *et al.* (1979) have pointed out that this fungus is a member of the taxon *Coprinus cinereus*. For fruiting, an agar block with mycelium (2 mm $\times$ 2 mm) was inoculated on the centre of CY-1 medium in Petri dishes 9 cm in diameter and incubated at 28 °C under a regime of 12 h light/12 h dark (Kamada & Takemaru, 1977*a*). The medium contains, per litre, 20 g glucose, 2 g peptone, 1 g yeast extract, 0.46 g KH_2PO_4 , 1 g K_2HPO_4 , 0.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 15 g agar. The light was supplied from white fluorescent lamps providing approximately 500 lx at dish level. When basidiocarp primordia had appeared on the mycelial mat, the lid of the Petri dish was replaced with an inverted beaker 15 cm in depth and 9 cm in diameter, which was

supported on the rim of the dish and held by pieces of adhesive tape. Under these culture conditions, basidiocarp maturation occurred 10 or 11 d after inoculation.

Preparation of stipe cell walls. Stipes (approx. 1 g fresh weight) were immersed in 20 ml McIlvaine buffer (pH 5.0) (McIlvaine, 1921) containing 0.01% (w/v) sodium azide to prevent bacterial contamination, homogenized in a Waring blender for 1 min at maximum speed, and then broken with a French press (Ohtake model 5615, Tokyo, Japan) operated at $23\,000\text{ lbf in}^{-2}$ (159 MPa). The cell-wall fraction was purified by low-speed centrifugations according to the method of Santos *et al.* (1979) with modification: it was washed twice with McIlvaine buffer (pH 5.0) containing 0.01% (w/v) sodium azide and 0.75 M-NaCl and then three times with the same buffer containing sodium azide only. All the above operations were carried out at 4 °C.

To obtain cell walls free of autolytic activity, the cell-wall suspension obtained as above was boiled for 10 min.

Dry weight determination of isolated cell walls. The cell-wall suspension was boiled for 10 min to terminate autolysis. The cell walls were washed three times with distilled water and then with increasing concentrations of ethyl alcohol, to absolute alcohol, by low-speed centrifugations. Finally, the pellet was dried to constant weight at 37 °C.

Autolysis of isolated cell walls. Cell walls obtained from approximately 1 g fresh weight of stipes were suspended in 10 ml McIlvaine buffer (pH 5.0) containing 0.01% (w/v) sodium azide, and incubated with continuous shaking (50 strokes min^{-1}) at 37 °C. The dry weight of cell walls per g fresh weight of stipes ranged from 11.3 mg to 24.1 mg according to the stage of stipe elongation. To analyse products liberated by autolysis, the suspension was centrifuged at low speed and 1 ml of the supernatant was withdrawn.

Fractionation of cell-wall polysaccharides. Cell-wall polysaccharides other than chitin were fractionated into four fractions according to a minor modification of the method of Wessels (1965) as previously described (Kamada & Takemaru, 1977b): fraction I is a polysaccharide extracted with 0.5 M-acetic acid at 75 °C, and fractions II, III and IV correspond, respectively, to S-glucan, an alkali- and acid-soluble polysaccharide, and R-glucan of *Schizophyllum commune*, on the basis of solubility characteristics.

Gel chromatography. A 1 ml portion of soluble autolytic products was boiled for 10 min to terminate autolysis and applied to a Bio-Gel P-2 column (Bio-Rad, 200–400 mesh; $1.1 \times 80.0\text{ cm}$) previously equilibrated with McIlvaine buffer (pH 5.0) containing 0.01% (w/v) sodium azide. The elution rate was 4.3 ml h^{-1} . Fractions of 1 ml were collected and analysed for sugar content.

Gas chromatography. The soluble autolytic products were boiled for 10 min to terminate autolysis and dried *in vacuo* at 35 °C. The samples were hydrolysed in 3 M-HCl at 100 °C for 3 h and then neutralized with NaOH according to the method of Griggs *et al.* (1971). The resulting monosaccharides were converted to the alditol acetate derivatives and analysed using a gas-liquid chromatograph (Hitach model 163, Tokyo, Japan), according to the procedure of Griggs *et al.* (1971).

Chemical assays. Total carbohydrate content was determined with the anthrone reagent (Fairbairn, 1953) using glucose as standard. Protein was measured by the Lowry method, using bovine serum albumin as standard.

RESULTS

Autolysis of isolated cell walls

Under the culture conditions described in Methods, stipe elongation during basidiocarp maturation proceeds in a synchronous manner (Kamada & Takemaru, 1977a). The rate of stipe elongation increased up to 18 h after the start of illumination on the day of maturation and then decreased rapidly. Elongation ended by 24 h. Cell walls isolated from stipes at 15 h showed a high autolytic capacity. In a 24 h incubation period, they lost 55% of their dry weight, and 400 µg neutral sugar and 80 µg protein equivalents per mg of their dry weight were detected in the supernatant of the reaction mixture. Gas chromatography revealed that the major component of the carbohydrate liberated by autolysis was glucose (86.2%), with small amounts of *N*-acetylglucosamine (7.0%), galactose (3.1%), rhamnose (1.6%), arabinose (1.2%) and mannose (0.9%). This indicates that neutral sugar is a major product of autolysis. Boiled cell walls released only a negligible amount of neutral sugar (Fig. 1), showing that the release of the sugar is enzymic. In the pH range 2.2 to 8.0, sugar release was most rapid at pH 5.0.

The time-course study revealed that the rate of autolysis declined as autolysis proceeded (Fig. 1). In an attempt to understand the cause for the decline, autolysis was followed after the addition of boiled cell walls to the reaction mixture which had been incubated for 12 h. The boiled cell walls clearly increased the autolysis rate (Fig. 1), suggesting that substrate

Fig. 1. Autolysis of isolated cell walls of the *Coprinus macrorhizus* stipe. Freshly prepared (○) and boiled (●) cell wall samples (13 mg dry wt) from stipes 15 h after the start of illumination on the day of basidiocarp maturation were incubated at 37 °C in McIlvaine buffer (pH 5.0) containing 0.01% (w/v) sodium azide, and the liberation of neutral sugar was followed as described in Methods. In another experiment, after 12 h incubation of freshly prepared cell walls (13 mg dry wt), boiled cell walls (13 mg dry wt) were added to the reaction mixture, and the release of the sugar was followed (■). Sugar release is expressed as µg anthrone-positive materials released per mg dry weight of freshly prepared cell walls.

Fig. 2. Bio-Gel P-2 profiles of the sugar liberated after autolysis for 0 h (a) and 3 h (b). A 1 ml portion of the product liberated from the stipe cell walls at the 15 h stage was applied to a Bio-Gel P-2 column and eluted with McIlvaine buffer (pH 5.0) containing 0.01% (w/v) sodium azide. Fractions of 1 ml were collected and analysed with the anthrone reagent. Vo, void volume; Gen, gentiobiose; Glc, glucose.

limitation was responsible for the reduction in autolysis rate. However, the improvement appeared incomplete; the sugar release during the first 3 h after the addition of the boiled cell walls was approximately 67% of that during the first 3 h of autolysis proper (Fig. 1). This may have been due to insufficient access of the boiled cell walls to autolytic enzymes, or to a progressive loss of enzyme activity with time. A similar phenomenon has been reported for cell wall autolysis in *Aspergillus nidulans* (Polacheck & Rosenberger, 1975).

Polymeric nature of autolytically liberated sugar

The soluble products obtained after 30 min autolysis were chromatographed on a Bio-Gel P-2 column (Fig. 2). Polymeric (degree of polymerization >10), dimeric and monomeric

Table 1. *Amounts of polymeric, dimeric and monomeric products liberated during autolysis*

Cell walls isolated from stipes 15 h after the start of illumination on the day of basidiocarp maturation were autolysed for the times indicated, and the liberated products were separated by Bio-Gel P-2 column chromatography as described in Methods. Results are expressed as μg glucose equivalents per mg dry weight of the cell wall.

Time (h)	Polymers*	Dimers	Monomers	Total
0.5	24.2	2.3	28.7	55.2
1	24.7	2.6	57.8	85.1
3	5.5	5.5	169.3	180.3
6	8.8	0	283.4	292.2
12	10.9	0	353.1	364.0
24	4.0	0	396.3	400.3

* Degree of polymerization > 10 .

Table 2. *Change in the composition of the cell-wall polysaccharides other than chitin by autolysis*

Cell walls isolated from stipes 15 h after the start of illumination on the day of basidiocarp maturation were fractionated before and after 24 h autolysis, and the sugar contents in the respective fractions were determined as described in Methods. Results are expressed as μg glucose equivalents per mg dry weight of the cell walls before autolysis.

Fraction	Before autolysis	After autolysis
I	21.4	5.4
II	47.1	94.1
III	130.1	45.9
IV	303.5	38.9
Total	502.1	184.3

Fig. 3. Relationship between the initial rate of autolysis (O) and stipe elongation (histogram) of *Coprinus macrorrhizus*. Autolysis is expressed as μg anthrone-positive materials released per stipe during the first 3 h of autolysis at 37°C in McIlvaine buffer (pH 5.0). The average of at least three measurements is given for each plot. Elongation rate is based on the data of Kamada & Takemaru (1977a). Since the size of basidiocarps varied between culture dishes, basidiocarps of average size (stipe length at the 6 h stage was approximately 12 mm) were selected for the measurements. The abscissa shows hours after the start of illumination on the day of basidiocarp maturation.

components were detected. The time-course study showed that the amount of the polymeric component decreased during the first 3 h of autolysis, whereas that of the monomeric component gradually increased as the reaction proceeded (Table 1). The dimeric component had completely disappeared by 6 h. These results suggest that both exo- and endo-mechanisms of glucan hydrolysis are involved in the autolysis reaction.

Polysaccharide composition of autolysing cell walls

The stipe cell-wall polysaccharides other than chitin are separable into at least four components (Kamada & Takemaru, 1977*b*). To investigate which components were solubilized by autolysis, the amounts of the four components in the cell walls before and after autolysis were determined (Table 2). Fractions I, III and IV were highly susceptible to autolytic enzymes, whereas fraction II was not.

Relation between the rates of autolysis and stipe elongation

Stipe cell walls from various stages of elongation were allowed to autolyse, in order to examine the relation between autolysis and stipe elongation. Changes in the initial rate of autolysis paralleled changes in the elongation rate, although the decrease in elongation rate after the 18 h stage appeared to progress slightly ahead of the decrease of the autolysis rate (Fig. 3).

DISCUSSION

A role for cell-wall lytic enzymes in hyphal growth has been suggested (Bartnicki-Garcia & Lippman, 1972; Bartnicki-Garcia, 1973). Supporting evidence that autolytic enzymes are involved in apical growth and branching of vegetative hyphae has been obtained in a variety of filamentous fungi (Mahadevan & Mahadkar, 1970; Polacheck & Rosenberger, 1975; Fèvre, 1977). The present study showed a positive relation between the initial rate of autolysis of stipe cell walls and the rate of stipe elongation, suggesting that autolytic enzymes are involved in the mechanisms of the elongation of stipe cells.

It has been reported that the autolysis of the hyphal wall in *Saprolegnia monoica* is mostly due to an endo-glucanase (Fèvre, 1977). In the stipe cell wall of *C. macrorhizus*, however, both exo- and endo-glucanases must be involved in autolysis, because both monosaccharide and polysaccharide was liberated during the initial stages of autolysis, and the polysaccharide was converted to monosaccharide as the reaction proceeded (Fig. 2, Table 1). Thus there may be a difference in growth mechanism between vegetative hyphae and stipe cells. Involvement of both exo- and endo-glucanases in the autolysis of cell wall has been reported in the higher plant *Zea mays* (Huber & Nevins, 1979, 1980, 1981).

In *Aspergillus nidulans*, only 3% of the hyphal wall was solubilized during 48 h autolysis (Polacheck & Rosenberger, 1975), and in *Saprolegnia monoica*, less than 3.6 µg sugars per mg of hyphal wall were released during 18 h (Fèvre, 1977). On the other hand, cell walls from rapidly elongating stipes of *C. macrorhizus* lost as much as 55% of their dry weight during 24 h autolysis. This high autolytic capacity may be a reflection of the conspicuously high rate of stipe elongation in this fungus (Kamada & Takemaru, 1977*a*).

The present investigation showed that the polysaccharide components of the stipe cell wall, fractions I, III and IV, were highly susceptible to autolytic enzymes, whereas fraction II was not (Table 2). The amount of fraction II increased during autolysis (Table 2), suggesting that some cell-wall component(s) might be converted to fraction II by structural modification during autolysis. Chitin hydrolysis appeared to be small, since only a small amount of *N*-acetylglucosamine and/or its polymer was detected in the products of autolysis. These results are consistent with our previous results that the amounts of fraction I, III and IV markedly decrease per unit stipe length as stipe elongation proceeds, whereas that of fraction II is almost constant and that of chitin decreases only slightly (Kamada & Takemaru, 1977*b*). These results also show that fraction II as well as chitin might function as a skeletal component of the stipe cell wall. Further understanding of the function of the respective cell-wall components must await the results of investigations, which are in progress, into their molecular structure.

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