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Enzymic Degradation of Septa in Hyphal Wall Preparations from a Monokaryon and a Dikaryon of *Schizophyllum commune*

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SUMMARY

Hyphal wall preparations of *Schizophyllum commune* were treated with enzymes and the dissolution of wall components and the degradation of septa were simultaneously recorded. The breakdown of the alkali-insoluble R-glucan (β-1,3, β-1,6-glucan) by R-glucanase was not influenced by the presence of chitinase but the breakdown of chitin by chitinase was stimulated by R-glucanase. The combination of R-glucanase and chitinase also had a synergistic effect on crosswall degradation. This indicates that the crosswalls contain both chitin and R-glucan, the chitin probably embedded in R-glucan. S-glucan (α-1,3-glucan), a prominent component of the lateral walls, is apparently absent from the crosswalls. The septal swellings consist of material that is alkali soluble but different from S-glucan. Crosswalls in hyphal wall fragments from a dikaryon were much more resistant to enzymic dissolution than those in hyphal wall fragments from a monokaryon. Such a difference could not be noted in the dissolution of wall components. The results are discussed with regard to their significance for sexual morphogenesis in *Schizophyllum commune*.

INTRODUCTION

In basidiomycetes such as *Schizophyllum commune* the dikaryon, which normally bears fruiting bodies, is produced by the mating of two monokaryons with different alleles in two incompatibility factors, *A* and *B*. After nuclear exchange the invading nuclei migrate through the mycelium of each mate prior to the establishment of the binucleate state in apical hyphae. Nuclear migration is a transient process in the formation of the dikaryon but it occurs continuously in common-*A* heterokaryons produced by mating monokaryons with different *B* factors but the same *A* factor. The same happens in homokaryons which carry a mutation in the *B* factor (Raper, 1966).

Although nothing is known about the motile forces responsible for nuclear migration, electron micrographs have revealed that the complex septal apparatus which prevents passage of nuclei from cell to cell (Girbardt, 1962) is disintegrated simultaneously with the initiation of nuclear migration (Giesy & Day, 1965; Jersild, Mishkin & Niederpruem, 1967; Koltin & Flexer, 1969; Raudaskoski, 1973; Marchant & Wessels, 1974). Biochemical studies with *S. commune* (Wessels & Niederpruem, 1967; Wessels, 1969*a*) suggested that an increase in enzymes hydrolysing the alkali-insoluble β-1,3, β-1,6-linked glucan in the wall (R-glucan) is a factor in the process of septal dissolution. Subsequent studies employing isolated wall preparations showed that an R-glucanase preparation from *S. commune* in

combination with chitinase could indeed completely dissolve the septa but not the longitudinal walls (Janszen & Wessels, 1970).

R-glucanase also increases sharply in a dikaryon of *S. commune* after exhaustion of the glucose supply in the medium and concomitant with expansion of the pilei of the fruiting bodies (Wessels, 1966; Wessels & Niederpruem, 1967). Although R-glucan is broken down, septal dissolution has never been shown to be characteristic for this stage of the life cycle. A possible explanation for the stability of septa in the dikaryon, even in the presence of high R-glucanase activities, would be that these septa are resistant to enzymic attack.

The present study was undertaken to obtain quantitative information about the role of R-glucanase and chitinase in septal dissolution and to compare the susceptibility to enzymic degradation of septa in the monokaryon and the dikaryon.

METHODS

Organisms and culture conditions. Strains 699 (*A41B41*) and 845 (*A51B51*) of *Schizophyllum commune* were used. The dikaryon was established by mating the two strains at 33 °C, thus preventing fruit-body formation. The mycelia were fragmented in a Waring blender (1 min at half speed) and the suspensions were used to inoculate Fernbach flasks (2 l) containing 100 ml liquid minimal medium (Wessels, 1965). The cultures were incubated under standing conditions at 25 °C with continuous fluorescent illumination.

Hyphal wall preparations. The mycelia were harvested on nylon cloth, washed with water and then with 50 mM-KH_2PO_4–NaOH buffer (pH 7·0) and frozen. Virtually complete breakage of hyphal compartments was achieved with an X-Press (Biotec, Stockholm, Sweden) at about −25 °C. The wall fragments were washed on the centrifuge at 4 °C (5 min, 3000 *g*), five times with 50 mM-phosphate buffer (pH 7·0) and then four times with water. Between the third and fourth buffer wash and between the first and second water wash, the wall suspensions were treated with a sonifier (Branson, B12) for 1 min. The washed walls were freeze-dried and stored at −25 °C. Before use the walls were resuspended in buffer by brief sonification.

Degradation and assay of wall components. Enzymic degradation was carried out in 1 ml McIlvain buffer (14·2 mM-citric acid – 35·8 mM-Na_2HPO_4, pH 5·6) containing 10 mg hyphal walls. Incubations were done at 30 °C for 24 h in closed tubes. Each tube received four drops of toluene to prevent bacterial growth. The enzymes used were crude R-glucanase (Wessels, 1969*b*) and chitinase (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.).

After incubation, the reaction mixtures were centrifuged and the pellets washed with water. The amount of S-glucan, R-glucan, and chitin (insoluble hexosamine) in the pellets was then determined as described by de Vries & Wessels (1973). In addition, the R-glucan fraction solubilized by treatment with 0·55 N-HCl for 1 h at 100 °C was hydrolysed further with 6 N-HCl at 100 °C for 6 h, followed by determination of hexosamine. This fraction was designated as 'soluble hexosamine'.

Microscopic methods. After enzymic treatment the walls were washed with water and centrifuged to form a pellet prior to fixation for 30 min in 1·5 % aqueous potassium permanganate. The pellet was washed with water and dehydrated in an alcohol series without resuspending it, and was retained in this form for the remainder of the processing. Following dehydration the material was washed in 1,2 epoxy propane and embedded in Epon.

For light microscopy, 1 μm sections were cut with a glass knife on an LKB Ultratome and transferred with a drop of water to a glass slide which was then heated on a hot plate (70 °C) for 10 min. The sections were stained on the hot plate for 2 to 3 min with a 1:1 (v/v) mixture

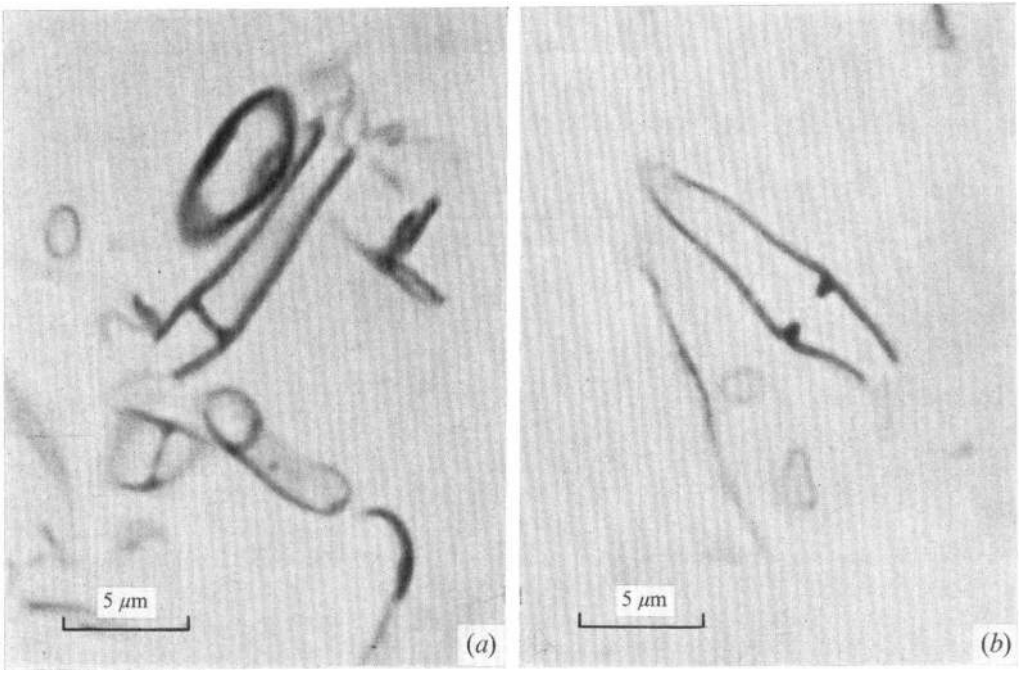

Fig. 1. Non-dissolved (*a*) and dissolved (*b*) septa as seen in 1 μm sections of hyphal wall preparations of *Schizophyllum commune* (699) stained with toluidine–borax.

of 1 % toluidine blue and 1 % borax. The stained sections were washed in water, dried, and mounted in Entellan (Merck). Septa were scored as dissolved if a hole could be seen in the septa without any material visible in the opening (Fig. 1). Septa without a hole visible or with only a thinning in the middle, representing either undissolved septa or non-median sections through dissolved septa, were scored as undissolved. This technique provides a gross underestimate of the actual level of septal dissolution, but is the most standard method for comparative results. Each dissolution percentage was based on examination of 125 to 250 septa.

For electron microscopy, the sections were cut with a diamond knife on an LKB Ultratome or a Cambridge Huxley Mark II Ultramicrotome, picked up on uncoated grids and viewed at 60 kV in a Philips EM 300 or A.E.I. EM 6G microscope.

RESULTS

Role of R-glucanase and chitinase in septal dissolution

Hyphal wall fragments of strain 699 of *S. commune* were incubated with R-glucanase and chitinase alone and in combination. Table 1 shows that chitinase alone removes 20·8 % of the insoluble hexosamine (chitin) from the wall, but none of the other components, including the soluble hexosamine fraction, is affected. This indicates that this soluble hexosamine is not derived from chitin but is a component of another wall fraction. Also, chitinase alone does not dissolve any septa in addition to the low percentage seen in untreated hyphal walls. A significant increase in dissolved septa is observed after treatment with R-glucanase alone. This treatment removes a substantial amount of R-glucan (41·1 %) and soluble hexosamine (27·9 %) and a small amount of insoluble hexosamine (8·7 %) from the wall.

Table 1. *Effect of R-glucanase and chitinase on solubilization of wall components and dissolution of septa in a hyphal wall preparation of Schizophyllum commune* (699)

Hyphal walls were prepared from 4·5-day-old cultures and incubated (10 mg/ml) with R-glucanase (1 mg/ml) and/or chitinase (1 mg/ml) at 30 °C for 24 h.

	Amount left after treatment*				
			Hexosamine		
	S-glucan	R-glucan	(Soluble)	(Insoluble)	Septa dissolved†
No enzymes	30·9	32·6	4·3	9·1	1·0
R-glucanase	29·3	19·2	3·1	8·3	7·5
Chitinase	30·8	32·6	4·3	7·2	1·0
R-glucanase + chitinase	27·4	18·8	2·7	5·2	36·9

* Given as percentage of the dry weight of the original wall.
† Given as percentage of the total number of counted septa in 1 μm sections.
All differences observed are significant at the 0·1 % level.

As R-glucanase solubilizes a large part of the soluble hexosamine it may belong to the R-glucan complex. Two facts should be recognized about the breakdown of insoluble hexosamine by the R-glucanase preparation. First, short term experiments, in which the appearance of reducing sugar was measured after incubation of crustacean chitin with the R-glucanase preparation, revealed virtually no chitinase activity (Wessels, 1969*b*). A more careful measurement of the amount of residual chitin after prolonged incubation did show the presence of significant chitinase activity. Incubation of 2·5 mg chitin with 1 mg R-glucanase or chitinase in 1 ml McIlvain buffer at 30 °C for 24 h, followed by determination of glucosamine in the residue, showed 12 % and 70 % breakdown of the chitin, respectively. Second, the R-glucanase preparation increases the susceptibility of chitin in the wall to chitinase, as was evident from the combined use of R-glucanase and chitinase.

The combined action of R-glucanase and chitinase removes about the same amounts of R-glucan and soluble hexosamine from the walls as R-glucanase alone (Table 1). For the insoluble hexosamine, however, a synergistic effect can be noted. Whereas R-glucanase and chitinase separately remove 8·7 and 20·8 %, respectively, of insoluble hexosamine from the walls, in combination they remove 42·8 %. Even more conspicuous is the synergistic effect on the dissolution of septa; the combination of enzymes is 4 to 5 times more effective than when these enzymes are used separately.

Pretreatment of the walls with chitinase had virtually no effect on the breakdown of R-glucan by R-glucanase. On the other hand, after treatment of the walls with R-glucanase, chitinase was able to lower the chitin to the same extent as in combination with R-glucanase. For septal dissolution it was found that successive treatment with enzymes, irrespective of the sequence, was as effective as using the enzymes in combination.

None of the enzymes appreciably attacked the alkali-soluble S-glucan fraction (Table 1). The small breakdown noticeable with R-glucanase may be due to the presence of a few other components in this fraction in addition to α-1,3 glucan (Wessels *et al.* 1972) and the presence of contaminating enzyme activities in the R-glucanase preparation.

From these results it can be concluded that both the longitudinal walls and the septa contain R-glucan and chitin and that R-glucan apparently protects the chitin from being attacked enzymically. S-glucan does not seem to play a role in the maintenance of integrity of the septa since its degradation is not necessary for septal dissolution to occur.

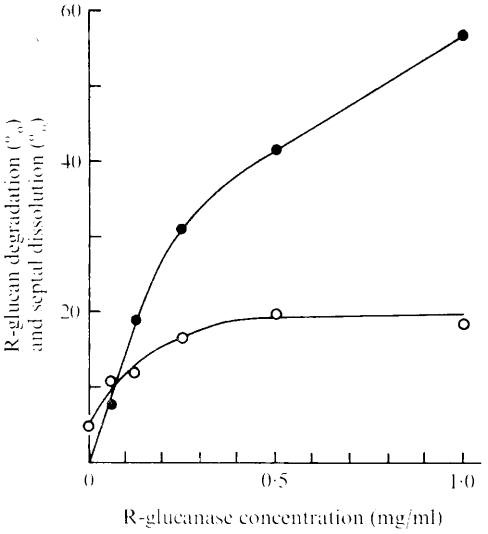

Fig. 2. Effect of R-glucanase concentration on R-glucan degradation (●) and septal dissolution (○) in a hyphal wall preparation of *Schizophyllum commune* (699). Hyphal walls (10 mg/ml) were incubated with chitinase (1 mg/ml) and varying concentrations of R-glucanase at 30 °C for 24 h.

R-glucanase concentration and septal dissolution

Hyphal wall fragments of strain 699 were incubated with a constant concentration of chitinase and varying concentrations of R-glucanase. Both degradation of R-glucan and septal dissolution were recorded. Figure 2 shows that a plateau in septal dissolution percentage is reached at an R-glucanase concentration of about 0·25 mg/ml, although increasing concentrations still effectively dissolve more R-glucan. It thus appears that R-glucan and probably chitin in these dissolved septa was more susceptible to degradation than are the same components in the longitudinal walls. This may be due to the presumed absence of S-glucan in these septa since it was found that extraction of S-glucan from the hyphal wall preparation with alkali increases the susceptibility of R-glucan and chitin to enzymic degradation about fourfold.

Septal dissolution in wall fragments derived from a monokaryon and a dikaryon

Isolated wall fragments obtained from the monokaryon (699) and the dikaryon (699/845) grown for 4·5 and 9 days were incubated with chitinase and R-glucanase. Table 2 shows that septa from the dikaryon were much more resistant to enzymic dissolution than those of the monokaryon. The difference is particularly clear at the lower R-glucanase concentration but is also significant at the higher concentration. It appears that little difference exists in the susceptibility of septa in young and old hyphae of both the monokaryon and the dikaryon. There is no correlation between septal dissolution and degradation of wall components at a given R-glucanase concentration. In fact, the enzymic treatment dissolved more R-glucan and chitin from the walls of the dikaryon than from those of the monokaryon.

To test the possibility that the enzymic treatment also effectively dissolved R-glucan and chitin from the septa of the dikaryon but failed to dissolve the septa because of the presence of S-glucan, the enzymically treated wall fragments were subsequently extracted with alkali. Table 3 shows that an increase in the septal dissolution percentage occurred due to the alkali treatment, but that a difference between the monokaryon and the dikaryon can still

Table 2. *Dissolution of wall components and septa in hyphal wall preparations of monokaryotic* (699) *and dikaryotic* (699/845) *mycelium of Schizophyllum commune*

Hyphal wall preparations were derived from mycelium grown for 4·5 and 9 days and incubated (10 mg/ml) with chitinase (1 mg/ml) and R-glucanase at 30 °C for 24 h.

Walls from	Culture age (days)	R-glucanase concentration (mg/ml)	Wall component dissolved (%)			Septa dissolved* (%)
			S-glucan	R-glucan	Chitin	
699	4·5	0·5	—	39·0	—	(3·0) 19·3
699/845	4·5	0·5	—	52·8	—	(0·0) 2·4
699	4·5	2·0	13·1	76·3	73·9	(3·0) 35·9
699/845	4·5	2·0	14·9	89·1	80·2	(0·0) 14·2
699	9	2·0	14·1	77·8	68·6	(3·0) 33·3
699/845	9	2·0	6·6	88·1	83·3	(2·0) 6·9

* Percentages of septa dissolved after treatment with buffer alone are given in parentheses. Differences between the monokaryon and dikaryon in each set are significant at the 0·1 % level.

Table 3. *Dissolution of septa by enzymes and alkali in hyphal wall preparations of Schizophyllum commune*

Hyphal wall preparations from 4·5-day-old mycelium were incubated (10 mg/ml) with chitinase (1 mg/ml) and R-glucanase (2 mg/ml) at 30 °C for 24 h. After washing with water the hyphal fragments were embedded in 2 % agar. The embedded walls were either directly fixed and processed for ultramicrotomy or first extracted with 1 N-KOH at 60 °C for 20 min.

Walls from	Septa dissolved (%)*	
	Enzymic treatment	Subsequent alkali treatment
699	46·2	53·2
699/845	29·7	35·8

* Differences between the monokaryon and the dikaryon are significant at the 0·1 % level.

be observed. This indicates that the septa in the wall fragments of the dikaryon, still entire after enzymic treatment, are not solely composed of S-glucan. The results rather suggest that the alkali-insoluble components in the septa of the dikaryon are well protected against enzymic degradation.

Electron microscopy of septal dissolution in hyphal wall fragments

Figure 3(*a*) illustrates the appearance of the septum in an isolated wall fragment of strain 699 immediately after isolation. The septal crosswall comprised three layers, two peripheral

Fig. 3. Sectioned hyphal wall fragments of *Schizophyllum commune* (699) showing condition of the septum after various treatments. (*a*) No treatment, showing intact crosswall and dolipore swelling. (*b*) Incubated with R-glucanase (1 mg/ml) and chitinase (1 mg/ml) for 30 min. Note the still intact septum (× 67100). (*c*) As (*b*), but incubated for 1 h; the dolipore swelling has been partially removed. (*d*) As (*b*), but incubated for 24 h; complete degradation of the septum. (*e*) Incubated with R-glucanase (1 mg/ml) alone for 24 h; the septum is complete apart from the removal of the dolipore swelling. (*f*) Incubated with chitinase (1 mg/ml) alone for 24 h; this oblique section shows that the crosswall and dolipore swelling have not been degraded. (*g*) Incubated with buffer alone for 24 h; there has been no autolysis of the septal structure. (*h*) Appearance of the septum after extraction with 1 N-KOH at 60 °C for 20 min; removal of the dolipore swelling and the outer S-glucan layer from the longitudinal walls, but the crosswall remains intact.

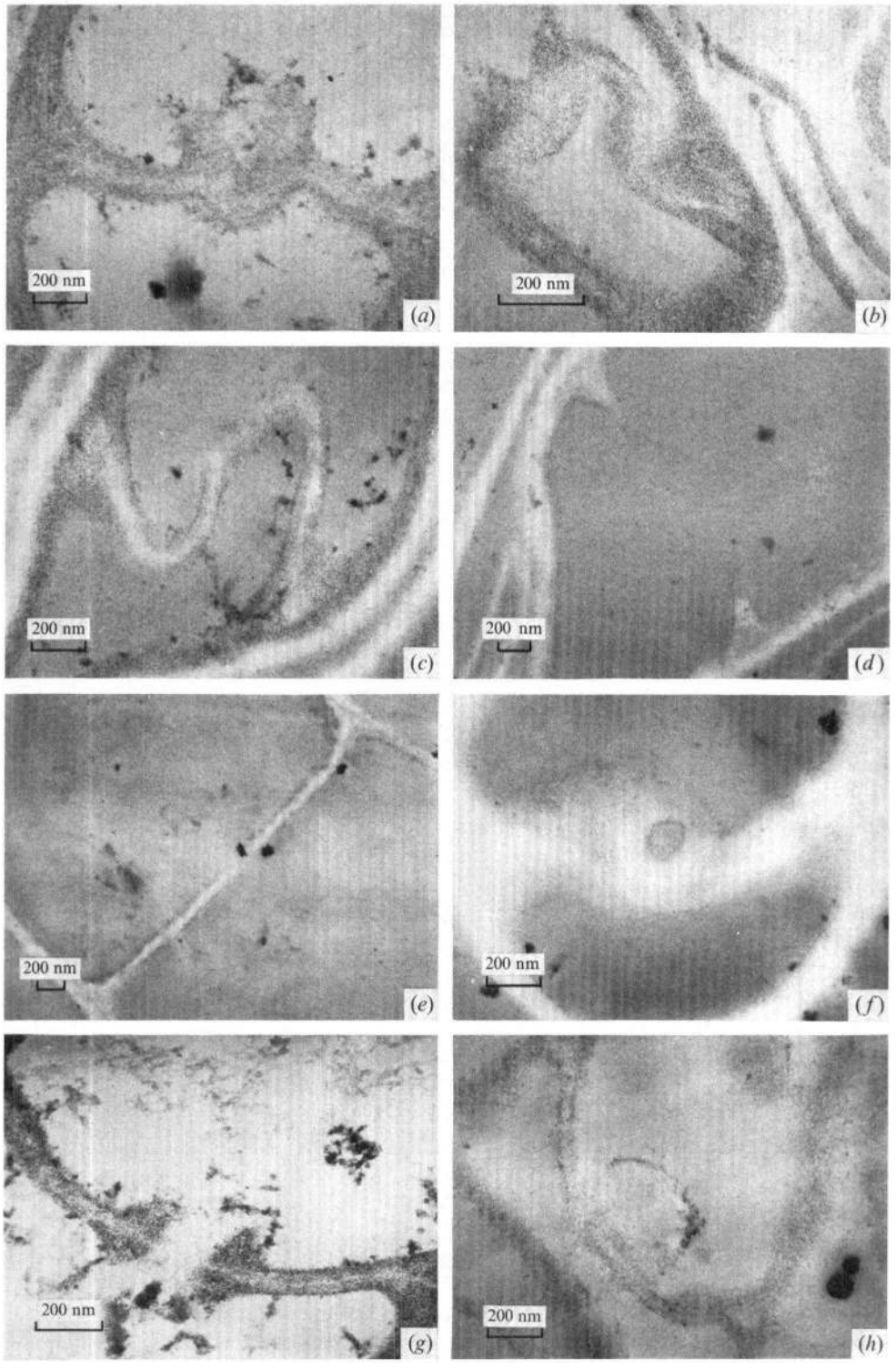

Fig. 3. For legend see opposite.

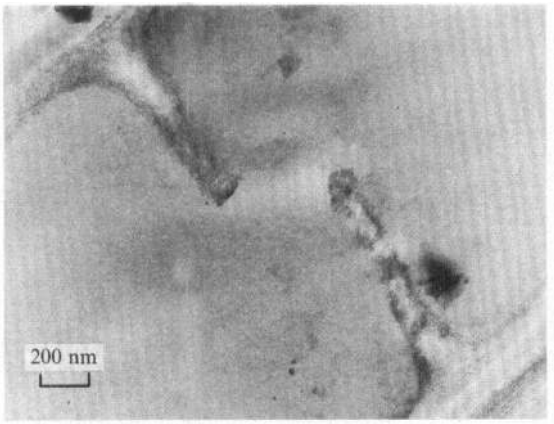

Fig. 4. Sectioned hyphal wall fragment of *Schizophyllum commune* dikaryon (699/845) incubated with R-glucanase (1 mg/ml) and chitinase (1 mg/ml) for 21·5 h. The dolipore swelling has been removed but the crosswall is resistant to degradation.

electron-opaque layers and a central electron-transparent layer; the dolipore swelling although somewhat diffuse around its margins was clearly intact. Figure 3(*b*) (*c*) and (*d*) shows the effect of 30 min, 1 h and 24 h incubation respectively on the hyphal wall fragments of strain 699 with R-glucanase (1 mg/ml) and chitinase (1 mg/ml) in combination. The 30 min incubation (Fig. 3*b*) produced no noticeable effect on the structures of the septal crosswall or dolipore swelling. After 1 h incubation (Fig. 3*c*) the dolipore swelling had been partially removed while the septal crosswall had lost a certain amount of its electron-opacity. Figure 3(*d*) illustrates the typical situation after 24 h incubation, with almost complete removal of the septal crosswall and no remaining evidence of the dolipore swelling. The wall material had also lost further electron-opacity and remained electron-transparent. Treatment of isolated wall fragments with R-glucanase alone for 24 h (Fig. 3*e*) removed only the dolipore swelling in the majority of cases but degradation of the crosswall was not observed. In parallel with this, a 24 h treatment with chitinase alone (Fig. 3*f*) neither removed the dolipore swelling nor degraded the septal crosswall. To establish that the cell wall preparation had no autolytic activity, walls were incubated in buffer alone for 24 h (Fig. 3*g*). No degradation of the dolipore swelling or septal crosswall was evident. Figure 3*h* shows the appearance of the septum after extraction with 1 N-KOH at 60 °C for 20 min. The removal of S-glucan was evident in the disappearance of the electron-transparent material at the outside of the longitudinal wall (Wessels *et al.* 1972). The crosswall, however, was not significantly changed in appearance except for the complete removal of the dolipore swelling.

Figure 4 shows a resistant septum in a hyphal wall preparation of the dikaryon after treatment with chitinase and R-glucanase for 21·5 h. While the dolipore swellings were removed in a high proportion of cases, the septal crosswalls were mostly left intact.

DISCUSSION

The results of this study confirm and extend earlier observations (Janszen & Wessels, 1970) on the susceptibility of septa of *Schizophyllum commune* to various treatments. The mechanical breakage of the walls followed by removal of the cytoplasm by extensive washing also removes all membranous material including the parenthosomes. The dolipore swellings, however, remain fairly intact. This refutes the contention of Thielke (1972) that the dolipore

swellings have to be regarded as membrane-bound structures filled with vacuolar fluid. On the other hand, the fact that treatment with alkali or R-glucanase removes the dolipore swellings but does not destroy the integrity of the crosswall shows their chemical composition to be different from that of the crosswalls. The removal of the septal swellings by the R-glucanase preparation, which is free of S-glucanase, indicates that these structures are not composed of S-glucan (α-1,3-glucan) which constitutes the outer layer of the longitudinal walls (Wessels *et al.* 1972). In addition to R-glucanase which specifically degrades the alkali-insoluble β-1,3, β-1,6 glucan (Wessels, 1969*b*), however, the R-glucanase preparation contains other enzymes, e.g. acting on β-1,3- and α-1,4-linked glucans. The exact chemical nature of the dolipore swellings therefore remains obscure. A recent ultrastructure study of septal dissolution *in vivo* (Marchant & Wessels, 1974) has indicated that the dolipore swellings are usually the first structures to disappear during disintegration of the septal apparatus.

The fact that R-glucanase and chitinase, in combination or in succession, are needed to dissolve the septa indicates that both R-glucan and chitin are present in these structures. At the concentrations of enzymes used, no difference could be noted in septal dissolution by reversing the order of R-glucanase and chitinase treatment. It was found, however, that a previous R-glucanase treatment facilitates chitin degradation by chitinase. This would imply that chitin is generally embedded in an R-glucan matrix. That a similar situation also holds for the crosswall is indicated by its ultrastructural appearance: two peripheral electron-opaque layers and a central electron-transparent layer. A major difference between the crosswalls and the longitudinal walls is the apparent absence of S-glucan in the former, at least in substantial amounts. This may be the reason that the septa are much more susceptible to enzymic degradation than the longitudinal walls.

Little can be said about the chemical basis for the decreased susceptibility of the crosswall in the dikaryon. The results of enzyme treatment followed by alkali treatment indicate that the enzymes failed to dissolve the alkali-insoluble portion of the crosswall and that the enzyme-treated crosswall is not solely composed of S-glucan. It cannot be excluded, however, that S-glucan was present in these septa and protected the alkali-insoluble portion against enzymic degradation. Other possibilities include a better protection of chitin by R-glucan, e.g. because of the presence of more R-glucan or a change in the structure of R-glucan making it less susceptible to R-glucanase.

The existence of a difference in the susceptibility of the septa to enzymic degradation between the monokaryon and the dikaryon examined in this study should be interpreted with caution. Whether such a difference is consistent with other monokaryons and derived dikaryons must be tested before definite conclusions can be reached. It has already been found that the septa in hyphal wall preparations from a *B*-factor mutant are very susceptible to enzymic degradation (unpublished observation) but it would also be very interesting to examine the susceptibility of septa in *A*-factor mutants, including a homokaryon with mutations in both the *A* and the *B* factor which mimics the heterokaryotic dikaryon in morphology (Raper, 1966). If the difference in septal stability indeed relates to the monokaryotic and dikaryotic condition, this suggests a simple model for explaining certain events during the formation of the dikaryon in a mating and during the formation of fruiting bodies. The correlation between high R-glucanase activity and septal dissolution in common-*A* heterokaryons (Wessels & Niederpruem, 1967) and *B*-factor mutants (Wessels, 1969*a*; Wessels & Koltin, 1972) suggests that an increase in R-glucanase activity is also instrumental in septal dissolution and nuclear migration after mating two fully compatible monokaryons. If the dikaryotic condition indeed entails the synthesis of a resistant septum, such a septum might be formed after an invading nucleus establishes this condition in an

apical cell. The first septum laid down after synchronous nuclear division would actually be maintained instead of being degraded. Since growing dikaryons show a low R-glucanase activity, it also appears that the formation of R-glucanase is switched off in the dikaryotic cells until the enzyme is produced again at the moment of glucose depletion in the medium and the onset of pileus expansion (Wessels, 1966; Wessels & Niederpruem, 1967). The high R-glucanase activity then serves the breakdown of R-glucan as a reserve substance in the hyphal walls of vegetative mycelium and stunted fruiting bodies (Wessels, 1965). Although a good ultrastructural study of the appearance of the septal apparatus in the vegetative mycelium of the dikaryon at this stage of the life cycle is not available, light microscopic observations do not indicate crosswall dissolution. The observed resistance to enzymic degradation of the crosswalls in hyphal wall preparations of the dikaryon might explain this. Whether the parenthosomes and dolipore swellings are affected at this stage has to be determined.

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