Hydrolysis of Walls and Formation of Sphaeroplasts in *Mucor rouxii*

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SUMMARY

Walls from *Mucor rouxii* were partially lysed by an enzymatic complex present in filtrates from cultures of *Streptomyces* sp. When germinated spores were incubated with the enzymatic complex in a protective medium, osmotically sensitive sphaeroplasts were formed. The enzyme responsible for the degradation of the wall showed optimum activity at pH 7.5 and 32 °C. It was susceptible to the presence of divalent cations. The enzymatic complex released hexosamines (but no *N*-acetyl hexosamines), neutral sugars and uronic acids from purified walls of the fungus. A wall-bound α -glucosidase was also rendered soluble by the enzyme. The enzymatic complex hydrolysed chitosan, but not chitin or mucoran, and hydrolysed the walls from only fungi belonging to the Mucorales. It is suggested that the hydrolytic capacity of filtrates from cultures of *Streptomyces* sp. developed in the presence of walls from *M. rouxii* was due to an enzyme (chitosanase), acting on chitosan but not on chitin.

INTRODUCTION

There is a considerable literature concerning the chemical components of the walls of fungi (for a review see Bartnicki-García, 1968), but little information on how these components are organized in the wall. The use of microbial enzymes which lyse the wall from fungi may be valuable in the study of this problem. In addition, these enzymes could aid the study of problems such as the isolation of cell membranes and mitochondria and the regeneration of walls by protoplasts. Among the micro-organisms which synthesize wall-lytic enzymes are several species of *Bacillus*, *Pseudomonas*, *Streptomyces* and *Microspora* (Salton, 1955; Horikoshi & Iida, 1958; Horikoshi, 1959; Furuya & Ikeda, 1960; Mitchell & Alexander, 1961, 1963; Ochoa, García Acha, Gason & Villanueva, 1963; Gascón & Villanueva, 1964; Tanaka & Phaff, 1965).

In the present paper the lysis of the wall from *Mucor rouxii* and the formation of sphaeroplasts from this fungus by the action of an enzymatic complex produced by a strain of *Streptomyces* sp. is described. The strain of *Streptomyces* used in this study was reported to hydrolyse walls from *Mucor ramannianus* by Jones, Bacon, Farmer & Webley (1968). A preliminary report has appeared elsewhere (Ramírez, Reyes & Ruíz-Herrera, 1970).

METHODS

Organisms. *Mucor rouxii* IM-80 was obtained from S. Bartnicki-García (University of California, Riverside, U.S.A.) and the strain of *Streptomyces* sp. no. 6 was kindly made available by D. Jones (Macaulay Institute for Soil Research, Aberdeen, Scotland). Other strains were from our culture collection.

Media. For growth of *Mucor rouxii* and fungi other than *Phytophthora capsici*, complex media containing yeast extract, peptone and glucose (YPG) or maltose (YPM) media were used (Bartnicki-García & Nickerson, 1962*a*). For growth of *P. capsici* a medium of commercial vegetable juice (Campbell's V-8) diluted 3:10 and neutralized with 0.45% CaCO_3 was used. *Streptomyces* sp. no. 6 was grown in the following medium (g/l distilled water): K_2HPO_4 , 1.00; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.50; KCl, 0.50; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; NH_4NO_3 , 5.00; walls from *M. rouxii*, 1.00. Strains were maintained on slants of the same medium solidified with 2% agar.

Preparation of walls from fungi. Walls were prepared by the following procedure: *Mucor rouxii* was grown for 15 h in New Brunswick fermenters containing 30 l of YPG or YPM medium. The mycelium was recovered by filtration, thoroughly washed with water and freeze-dried with a Usifroid lyophilizer. Samples of the dried mycelium were ground with a Wiley cutting mill and homogenized with a Mixolabo (Jouan) homogenizer, each for 20 min. The temperature was kept below 5 °C by immersing the cup in an ice-salt bath. Walls were centrifuged at 5000 rev./min for 10 min, recovered and homogenized again. This procedure was repeated 12 times. Finally, when walls appeared free from cytoplasmic material as judged by microscopical observation of preparations stained with cotton blue they were freeze-dried and kept at -20 °C over silica gel. Walls from other fungi were prepared by a similar method, except that growth was obtained in Erlenmeyer flasks. A crude preparation of walls from *Phytophthora capsici* was obtained by grinding mycelia with a Potter-Elvehjem tissue homogenizer. The preparation was thoroughly washed by centrifugation.

Preparation of the enzymatic complex from Streptomyces sp. no. 6. The actinomycete was grown in shaken Erlenmeyer flasks containing the liquid medium described above for 8 to 10 days at 28 °C. The mycelium was eliminated by filtration. The medium was concentrated to 1/40 of its original volume by ultra-filtration, and it was dialysed at 4 °C for 20 h against 80 vol of 7 mM-tris-HCl buffer, pH 7.5. The crude enzymatic material was kept at -20 °C.

Preparation of germinated fungal spores. Erlenmeyer flasks containing YPG medium were inoculated with spore suspensions of the selected fungi and shaken at 28 °C until almost all spores had germinated (6 to 8 h). The contents were centrifuged or filtered through sintered glass filters, washed with 50 mM-tris-HCl buffer, pH 8.0, and resuspended in buffer. The suspensions were adjusted to an absorbance of 0.250 to 0.300 at 520 nm with a Maroc V (Jobin et Ivon) spectrophotometer.

Measurement of activity of the lytic complex from Streptomyces sp. no. 6. Activity was tested on suspensions of spores, walls, chitin and chitosan. It was monitored by the decrease in absorbance of the suspensions measured at 520 nm with a Maroc V spectrophotometer. Activity was also measured by release of *N*-acetyl hexosamines, hexosamines, total sugars, reducing material and uronic acids from one or several of the above mentioned substrates. Further details are given in the corresponding experiments.

Preparation of sphaeroplasts from Mucor rouxii. Germinated spores (10 mg) were re-suspended in 50 mM-tris-HCl, pH 8.0, containing 25% sucrose and incubated at 32 °C for 75 min with the enzymatic complex from *Streptomyces* sp. (60 to 150 µg of protein). Sphaeroplasts appeared after 15 min. Photographs of sphaeroplasts were taken with a Nachet microscope using interference phase-contrast technique.

Chemical methods. Total sugars were measured with the anthrone reagent (Dimler, Schaefer, Wise & Rist, 1952); reducing sugars by the method of Park & Johnson (1949); acetyl hexosamines as described by Reissig, Strominger & Leloir (1955); hexosamines by the modified method of Elson & Morgan (Ashwell, 1957); uronic acids with carbazole

Fig. 1. Effect of crude enzyme complex from *Streptomyces* sp. on germinated spores and walls from *Mucor rouxii*. Spore suspension (1.2 mg/ml) (a) or wall suspension (0.28 mg/ml) (b) were incubated at 32 °C in spectrophotometer cuvettes. The final volume was 3.0 ml in 7 mM-tris buffer at pH 7.5. Absorbance at 520 nm was measured at intervals. Open symbols: no enzyme added. Closed symbols: incubated with crude enzyme (60 µg of protein).

(Bitter & Ewins, 1961); proteins by the method of Lowry, Rosebrough, Farr & Randall (1951); and phosphate as described by Taussky & Shorr (1953).

Chemicals. Chitin was purified from commercial crustacean chitin as described by Ruíz-Herrera (1967). Chitosan and mucoran were a gift from S. Bartnicki-García. All other chemicals were of the highest quality available.

RESULTS

Initially several media were tested to assess the best for growth of the actinomycete. They included the base medium from Skujins, Potgieter & Alexander (1965) as modified by Jones *et al.* (1968), and several modifications of Czapek's mineral medium containing walls as the carbon source. CO₂ evolution was used as an index of growth. CO₂ evolved was drawn in a stream of air into 0.01 N-Ba(OH)₂ and determined by titration of residual base with 0.01 N-HCl. The medium described above gave the highest CO₂ values and was used in all further studies.

Accumulation of the lytic complex in the medium increased until the 8th or 9th day, and remained constant for about 3 days. Extracellular protein also accumulated in the medium and reached its maximal value (60 µg/ml) at the end of the 6th or 8th day of incubation. For these reasons, an incubation period of 8 days was chosen for further experiments.

Kinetics of wall lysis. When germinated spores or isolated walls from *Mucor rouxii* were incubated with the enzymatic complex from *Streptomyces*, turbidity of the suspensions decreased rapidly following characteristic kinetics. The rate of lysis decreased with time and became almost asymptotic (Fig. 1). The reduction in rate was not due to enzymatic inactivation, but to the existence of a resistant residue in the walls, since the residue obtained after a first enzymatic attack was not further hydrolysed when incubated with fresh enzymatic complex. Further, the lytic enzyme recovered after hydrolysing a sample of walls was still active and hydrolysed a fresh sample of walls added afterwards (Fig. 2). However chemical analysis showed that the solubilized and resistant fractions were similar in composition. This aspect will be further explained below.

Fig. 2. Effect of crude enzyme complex from *Streptomyces* sp. on walls from *Mucor rouxii*. (a) Walls (2.5 mg) were incubated in the conditions specified for Fig. 1. Absorbance was measured at 520 nm. Open symbols: no enzyme added. Closed symbols: 300 µg of protein of lytic enzyme added. (b) After 24 h of incubation, material from (a) was centrifuged. Lytic enzyme (300 µg of protein) was added to the residue; 2.5 mg of walls were resuspended in the supernatant. Both samples were again incubated and absorbance measured at intervals. Δ , Residue from (a) with fresh enzyme; $\blacktriangle$, supernatant from (a) with fresh walls.

Characteristics of the enzymatic complex from Streptomyces sp. no. 6. The effect of pH, temperature and divalent cations on lytic activity was tested. Activity of the enzyme depended on the pH and also on the composition of the buffer used. In the presence of cationic buffers, activity increased with pH, whereas with anionic buffers, activity decreased with pH. Results obtained with tris buffer (Fig. 3) show optimum activity at pH 7.6. The optimum temperature was 32 °C and activity decreased sharply at other temperatures (Fig. 4). All divalent cations tested were inhibitory. Complete inhibition was obtained when either Zn^{2+} , Co^{2+} or Hg^{2+} (1×10^{-3} M) were added to incubation mixtures, but Mn^{2+} , Ca^{2+} or Mg^{2+} added separately were less inhibitory (Fig. 5).

Chemical composition of material released from walls by the lytic complex. When walls from *Mucor rouxii* were incubated with the lytic complex from *Streptomyces* sp. no. 6, neutral sugars, hexosamines (but no *N*-acetyl hexosamines) and uronic acids were released to the supernatant. Release of all the components, and decrease in turbidity followed similar kinetics (Fig. 6). When the solubilized material was hydrolysed with 6 N-HCl, the amount of glucosamine increased about threefold, suggesting that, on average, the hexosamine-containing molecules had three residues of amino sugars.

In a further experiment, a sample of 30 mg of walls from *Mucor rouxii* was incubated at 32 °C for 24 h with 3 ml of enzymatic complex from *Streptomyces*. The final volume was adjusted to 15 ml, with 50 mM-tris at pH 8. The mixture was filtered and the insoluble residue was washed and freeze-dried. It appeared as a fluffy whitish material which weighed 6 mg. The chemical composition of the solubilized material and residue appears in Table I. The gross chemical composition of both fractions was very similar. However the supernatant contained slightly higher values of phosphate, total sugars and hexosamine, whereas the amounts of uronic acids and proteins were slightly higher in the insoluble residues. Recovery was 93 to 99%. The amount of phosphorus in the walls was much lower than the value reported for hyphal or yeast walls obtained from *Mucor rouxii* by Bartnicki-García & Nickerson (1962b). When walls obtained from mycelium grown in YPM medium were

Fig. 3

Fig. 4

Fig. 3. Effect of pH on the activity of lytic enzyme. Samples containing 1.0 mg of walls were incubated at 32 °C with crude enzyme (60 µg of protein) in 50 mM-tris at the indicated values of pH. The final volume was 2.0 ml. After 3 h, the sample was centrifuged and hexosamines and reducing material in the supernatant were measured. Closed circles: hexosamines in incubation medium (µg/ml); open circles: reducing material expressed as glucose (µg/ml).

Fig. 4. Effect of temperature on activity of lytic enzyme. Samples containing 1.0 mg of walls were incubated in 50 mM-tris-HCl buffer, pH 7.5. The final volume was 2.0 ml. After 3 h the sample was centrifuged and hexosamines and reducing material in the supernatant were measured. Closed circles: hexosamines (µg/ml); open circles: reducing material expressed as glucose (µg/ml).

Fig. 5

Fig. 6

Fig. 5. Effect of divalent cations on lysis of walls of *Mucor rouxii* by *Streptomyces* enzyme complex. Samples containing 1.5 mg of walls were incubated in spectrophotometer cuvettes with 60 µg of protein of crude enzyme in the presence of metallic chlorides (1 mM). The final volume was 3.0 ml in 50 mM-tris-HCl buffer, pH 8.0 and temperature 32 °C. Absorbance was measured at 520 nm. ○, With either 1 mM-Zn²⁺, 1 mM-Co²⁺ or 1 mM-Hg²⁺; ×, with Mn²⁺; ▲, with Mg²⁺; △, with Ca²⁺; ●, control, no divalent cation added.

Fig. 6. Release of components from walls of *Mucor rouxii* by *Streptomyces* enzyme complex. Walls (124 mg) were resuspended in 54 ml 7 mM-tris-HCl buffer, pH 7.5, and 8 ml of enzyme (4.8 mg of protein) was added. Starting at time of enzyme addition, 10 ml samples were taken and change of absorbance (%) was recorded (△) and the material was centrifuged and analysed; uronic acids (×), total sugars (●) and hexosamines (▲). A sample of each supernatant was hydrolysed with acid and hexosamines were measured (○). Data are expressed as percentage of each component released, taking as 100 % the amount of component present in an acid-hydrolysate obtained from 124 mg of *M. rouxii* walls.

Fig. 7. Hydrolysis of walls from *Mucor rouxii*, chitin and chitosan by enzyme preparation from *Streptomyces* sp. Samples (20 ml) of suspensions containing 1.0 mg of chitin, chitosan or walls per ml were incubated at 32 °C in 7 mM-tris buffer, pH 8.0, with or without enzyme (3.0 mg of protein). At intervals, samples were withdrawn and absorbance was measured. After centrifugation the supernatant was analysed for glucosamine released from chitosan and glucosamine and *N*-acetyl glucosamine released from chitin. ×, Absorbance of walls, chitin or chitosan suspensions incubated without enzyme; ▲, absorbance of chitin suspension with enzyme; ●, absorbance of chitosan suspension with enzyme; Δ, absorbance of wall suspensions with enzyme; ○, glucosamine released from chitosan.

Table 1. *Analyses of solubilized and insoluble material from Mucor rouxii* walls incubated with lytic enzyme

Walls (30 mg) were incubated at 32 °C for 24 h with 1.8 mg protein of enzyme in a final volume of 15 ml with 50 mM-tris, pH 8.0. The material was centrifuged, the residue recovered, washed, freeze-dried and weighed.

Compound	Supernatant		Residue	
	(mg)	(%)	(mg)	(%)
Total sugars*	6.975	29.06	1.230	20.50
Hexosamines*	10.500	43.70	2.478	41.30
Uronic acids*	2.164	9.01	0.762	12.70
Protein	2.063	8.59	0.720	12.00
Phosphate†	2.197	9.10	0.369	6.15
Total	23.899	99.46	5.559	92.65

* As the anhydrous form.

† As phosphorus.

incubated with the lytic complex, about 30 to 36% of total cell α -glucosidase was released to the supernatant after 21 h of incubation. According to data obtained in our laboratory, α -glucosidase is a wall-bound enzyme (Flores-Carreón, Reyes & Ruiz-Herrera, 1970).

Lysis of chitin, chitosan and mucoran (a polymer containing glucuronic acid, mannose, fucose and galactose; Bartnicki-García & Reyes, 1968) by the enzymatic complex from *Streptomyces* was tested. Lysis of chitin and chitosan was measured by release of hexosamine or *N*-acetyl hexosamine respectively and decrease in absorbance. Mucoran lysis was measured by release of reducing material. No activity was observed when mucoran was the substrate. When chitin was used, a slight decrease in absorbance was observed, but no hexosamines or *N*-acetyl hexosamines could be detected in the supernatant. When chitosan

Fig. 8. Morphology of germinated spores and sphaeroplasts from *Mucor rouxii*. The sphaeroplasts were obtained with *Streptomyces* enzymatic complex. (a) Germinated spores without enzyme. (b) Initial deformation of hyphae after enzymatic attack. (c) Formation of sphaeroplasts in 25 % sucrose. (d) After dilution of sucrose to 12.5 % germ tubes are pinched off. (e) Enlargement of sphaeroplasts maintained in 8.3 % sucrose. (f) Residue from protoplasts shocked with water.

was used, there was significant decrease in absorbance, and hexosamine was released to the supernatant (Fig. 7).

Formation of sphaeroplasts from Mucor rouxii. Sphaeroplasts were formed when germinated spores of *M. rouxii* were incubated with the enzymatic complex from *Streptomyces* in the presence of 25 % sucrose. These appeared as spherical bodies. Apparently the wall of the spore was more resistant to enzymatic attack than was the germ tube since the spore's content was emptied into the germ tube which rounded and swelled to become larger than the spore itself. The hyphal apex was the part most susceptible to lysis. When the sucrose medium was diluted, the size of sphaeroplasts increased, and both spore and enlarged germ tube formed a single spherical body, or they separated as two stable spherical bodies. As

Fig. 9. Degradation of walls from selected species of fungi by *Streptomyces* enzymatic complex. (a) Suspensions of hyphal walls (0.3 mg/ml) were incubated in 7 mM-tris buffer, pH 8.0, with 0.24 mg protein of crude enzyme. The final volume was 3.0 ml and temperature 32 °C. ●, *Psilocybe caerulescens*; ○, *Aspergillus flavus*; ×, *Phytophthora capsici*; △, *Phycomyces blakesleeanus*; ▲, *Mucor rouxii*. (b) Suspensions of germinated spores (3 mg/ml) were incubated with lytic enzyme as described above. ●, *Aspergillus oryzae*; ○, *Fusarium solani*; ×, *Cunninghamella echinulata*; △, *Rhizopus nigricans*; ○, *Rhizopus* sp.; ▲, *M. rouxii*.

the sucrose concentration was lowered to 10 to 12% the sphaeroplasts disintegrated suddenly and left a granular residue. Apparently there were no weaker points in the sphaeroplast envelope which permitted emptying. The appearance of sphaeroplasts maintained in several concentrations of sucrose is shown in Fig. 8.

Hydrolysis of the wall from selected fungi by the enzymatic complex from Streptomyces sp. Activity of the lytic complex was tested on walls isolated from hyphae of *Phycomyces blakesleeanus*, *Psilocybe caerulescens*, *Aspergillus flavus* and *Phytophthora capsici*, and from germinated spores of *Rhizopus* sp., *Rhizopus nigricans*, *Cunninghamella echinulata*, *Aspergillus oryzae*, and *Fusarium solani*. The results appear in Fig. 9, and show that walls from only Mucorales were susceptible to lysis by the enzymatic complex.

DISCUSSION

Our data suggest that the action of the enzymatic complex obtained from culture filtrates of *Streptomyces* sp. on the walls of *Mucor rouxii* was due to the action of one enzyme. Several observations are compatible with the hypothesis. First, it was observed that lytic activity of the filtrate increased with the age of the *Streptomyces* culture. When culture filtrates obtained after 2 and 8 days of incubation were mixed, no enhancement of wall lysis was observed which suggests that different lytic enzymes were not accumulated. Lysis of walls showed only one optimal pH in the several buffers tested and only one optimal temperature. When the complex was tested with walls from several fungi, it was observed that only Mucorales were susceptible to lysis. These are the only fungi tested which contain chitosan (Bartnicki-García, 1968). Moreover, no reducing material was released from walls obtained from *Aspergillus flavus* and *Psilocybe caerulescens*, both of which contain large amounts of glucans (Ruiz-Herrera, 1967; F. Esparza, personal communication). Jones *et al.* (1968) reported that culture filtrates from *Streptomyces* sp. grown on sporangiospore walls from *M. ramannianus* showed laminarinase activity which was induced by the laminarin-

type glucan present in such walls. Of the several polymers tested, only chitosan was hydrolysed by the enzymatic complex. Chitin and mucoran were resistant to hydrolysis and no *N*-acetyl hexosamines were released from isolated wall material. The observations suggest that hydrolysis of the wall is due to the action of an enzyme acting on chitosan but not on chitin. It is known that chitinase hydrolyses chitosan (Jeniaux, 1966), but an enzyme specific for chitosan (chitosanase) has not been described, and further experiments to characterize such an enzyme are necessary before reaching a final conclusion. If our hypothesis is correct, lysis of walls from *M. rouxii* would be different from the general pattern observed where more than one enzyme is required for the lysis of fungal and yeast walls (Skujins *et al.* 1965; Tanaka & Phaff, 1965; Villanueva, 1966), and it would be necessary to assume that, like the wall-bound α -glucosidase, the other components of the wall from *M. rouxii* are rendered soluble when the anchor material (chitosan), which gives the rigity to the wall, is solubilized. There were only slight chemical differences between the sensitive and resistant fractions of wall material. The simplest explanation is that, as the enzymatic reaction proceeds, the rate of hydrolysis changes because the susceptible substrate becomes less accessible to the enzyme, being protected by some other wall component. According to this hypothesis, given enough time, all the material except that completely inaccessible to the enzyme would be solubilized. Isolated walls were more resistant to the enzyme than the wall formed at spore germination. This suggests that the structures are different and that ageing of walls increases their resistance to enzymatic hydrolysis. We lack an explanation for this phenomenon, but manipulations of the wall, especially freeze-drying, may affect the organization of some resistant material, so making sensitive materials less accessible to the enzyme. The effect of divalent cations, and especially those of heavy metals, suggests that the enzyme contains a sulphydryl group necessary for activity.

Since walls of only Mucorales were lysed with the enzymatic preparation, specific wall-lysing enzymes may give information on taxonomic and phylogenetic relationships among fungi.

There are numerous reports on the preparation of sphaeroplasts (or protoplasts) from fungi by action of wall-lysing enzymes (Emerson & Emerson, 1958; Bachmann & Bonner, 1959; Mitchell & Alexander, 1963; Rodríguez Aguirre, Garcia Acha & Villanueva, 1964; Villanueva, 1966). Our microscopic observations revealed that the wall of the hyphal tip with the site of wall growth was the most susceptible part. Similar results have been described by Villanueva (1966).

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