CELL WALLS OF *PIRICULARIA ORYZAE*

I. SELECTIVE ENZYMOLYSIS OF *PIRICULARIA ORYZAE* WALLS BY WALL-LYTIC ENZYMES OF *BACILLUS CIRCULANS* WL 12

HIROSATO TANAKA, NAGAHIRO OGASAWARA,
TASUKU NAKAJIMA* AND KINJIRO TAMARI*

*Department of Agricultural Chemistry,
Niigata University, Niigata,
and*

*Department of Agricultural Chemistry,
Tohoku University, Sendai**

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Cell walls were prepared from *Piricularia oryzae* P₂. Lytic enzymes were produced when *Bacillus circulans* WL 12 was grown with *P. oryzae* cell wall as a sole source of carbon. Mode of lysis of *P. oryzae* cell walls was compared with the lysis of walls of *Aspergillus oryzae* and *Neurospora crassa*. Activities of β -1 $\rightarrow$ 3-glucanase, chitinase, and β -1 $\rightarrow$ 6-glucanase were demonstrated in the culture filtrate. Modes of lysis of cell walls of *P. oryzae* P₂ by a single and combined action of lytic enzymes were compared. Combination of β -1 $\rightarrow$ 3-glucanase and chitinase gave the most rapid and extensive lysis.

Pathogenicity of *Piricularia oryzae*, a group of rice blast molds, has hitherto been studied in our laboratory primarily from the stand point of toxins, piricularin and picolinic acid, produced by the pathogens (1, 2).

In order to expand the scope of our work on the host-parasite interactions, we recently started our work with cytological and biochemical approaches. Naturally, first contact between the host and the pathogen takes place at the surfaces of both organisms. We consider that the studies on the surface nature of *P. oryzae* and also of rice plants might give us some evidence which will help in our understanding of host-parasite interactions.

Our understanding on the cell walls of fungi is still meager when compared with our knowledge on the walls of yeasts and bacteria. Selective enzymolysis can be a fruitful approach for the study of fungal walls. Some works on this line have been published. Involvement of β -1 $\rightarrow$ 3-glucanase and chitinase, and especially their joint action in the enzymic lysis of a number of fungal species have been reported (3–6).

This paper describes studies on the cell walls of a strain of *Piricularia oryzae* P₂. Almost complete dissolution of mycelia of this rice pathogen occurred when a medium containing the mycelia was inoculated with *Bacillus circulans* WL 12. Nature of lytic activities was studied. Lytic enzymes were separated by column chromatography and lysis of walls by the single and combined action of these lytic enzymes was studied.

MATERIALS AND METHODS

Organisms. *Piricularia oryzae* P₂ was obtained from the National Institute of Agricultural Sciences. *Aspergillus oryzae* was obtained from the Institute of Applied Microbiology of the University of Tokyo, and *Neurospora crassa* IFO 6067 from the Institute of Fermentation, Osaka. Chitin-decomposing strain of *Streptomyces* CH9 was isolated from the soil collected in Niigata Prefecture. Strains of *Penicillium aculeatum* var. *apiculatum* Abe for the screening of luteic acid-producing strains were obtained from the Institute of Fermentation, Osaka, and the Institute of Applied Microbiology, University of Tokyo.

Media. Culture of *P. oryzae* P₂ was maintained on agar slant with Misato's medium (soluble starch 10 g, yeast extract 2 g, agar 15 g, in 1000 ml of water, autoclaved at 110° for 10 min). *Bacillus circulans* WL 12 was stored on agar slant with AWY medium (7). *Streptomyces* CH9 was kept on agar slant with asparagine medium (glucose 10 g, asparagine 0.5 g, KH₂PO₄ 0.2 g, agar 20 g, in 1,000 ml of water).

Laboratory-prepared yeast autolysate for the enzyme production was prepared as follows. Equal amounts by weight of water and commercial compressed baker's yeast were mixed. A small amount of toluene was added and the suspension was incubated at 55° for 3 days. The insoluble residue was collected by centrifugation, washed, and used as the autolysed and washed yeast (AWY). The supernatant was the original strength of laboratory-prepared yeast autolysate and it was diluted to the desired strength before preparing media. Vogel N-sucrose-yeast extract medium for the growth of *Piricularia oryzae* P₂ has the following composition. Stock solution (8): Trace element solution (×1,000 strength) contains citric acid 5 g, ZnSO₄·7H₂O 5 g, Fe(NH₄)₂(SO₄)₂·6H₂O 1 g, CuSO₄·5H₂O 0.25 g, MnSO₄·H₂O 0.05 g, H₃BO₃ anhydrous 0.05 g, Na₂MoO₄·2H₂O 0.05 g; 1 ml of chloroform added, and stored at room temperature. Stock solution (Vogel-N) (×50 strength) contained Na₃-citrate 150 g, KH₂PO₄ anhydrous 250 g, NH₄NO₃ anhydrous 100 g, MgSO₄·7H₂O 10 g, CaCl₂·2H₂O 5 g, H₂O 750 ml, 2 ml of chloroform added, and stored at room temperature. To make 1,000 ml of Vogel-N-sucrose-yeast extract medium, 1 ml of the trace element solution, 20 ml of the Vogel-N stock solution, 2.5 g of yeast extract (Kyokuto Pharmaceutical Co., Tokyo), and 20 ml of sucrose are mixed.

Mycelium production. Mycelia of *P. oryzae* P₂ for the preparation of

Fig. 1. Isolated cell walls.

The photograph was taken through a phase-contrast microscope.

cell walls and also for the use as the substrate in the lytic enzyme production by *Bacillus circulans* WL 12 were produced as follows. Conidia of *P. oryzae* were allowed to form on agar slants with Misato's medium for 2 weeks at 28°. In a 500-ml Sakaguchi flask, 100 ml of Vogel N-sucrose-yeast extract medium was placed, conidia obtained from a slant were inoculated into this flask with 100 ml of the above-mentioned medium, and the mold was allowed to grow on a shaker at 25° for 40 hr. One ml of the above culture was transferred to a Sakaguchi flask with the same medium and the mold was allowed to grow in the same condition as the seed culture. Mycelia were harvested after 40 hr of growth and stored frozen at -30°. Mycelia of *Aspergillus oryzae* and *Neurospora crassa* were made in a similar manner except for the conidia formation which was made on Czapek-agar slant for *A. oryzae* and Vogel N-sucrose-yeast extract medium for *N. crassa*.

Cell wall preparation. Forty grams in wet weight of the mycelium was mixed with 150 ml of glass beads of 300 μ average diameter (Otake Works, Tokyo) and 200 ml of Tween 80-EDTA-buffer consisting of 0.1% Tween 80 and 0.01 M ethylenediamine tetraacetate (neutralized before addition to the buffer in 0.2 M phosphate buffer of pH 7.0). The mixture was placed in a mixer (MX 80D, National Co., Tokyo) with a specially designed Teflon blades.

Fig. 2. Shadowed preparation of isolated cell walls.

A considerable contamination of metals occurred with ordinary stainless steel blades. Mycelia were disrupted in the mixer for about 30 min. Temperature was maintained below 10° by intermittent addition of powder Dry Ice. Breakage of mycelia and removal of cytoplasmic content from the broken mycelia were followed under phase-contrast microscope. Almost 100% disruption and removal of inner content of mycelia were achieved by this procedure. Glass beads were removed by decantation. Cell walls were washed several times with Tween 80-EDTA-buffer followed by repeated washings with deionized water. When the cell walls acquired floating characteristic (this interesting phenomenon will be discussed later in the section of experimental results), cell wall suspension was poured into a glass cylinder (5×90 cm) and allowed to stand at 3° overnight. During this time, most of broken glass settled to the bottom of the cylinder while cell walls remained suspended. Cell wall suspension was withdrawn by a siphon, centrifuged, and lyophilized. Walls of *A. oryzae* and *Neurospora crassa* were prepared in a similar manner. However, since the floating characteristic was only partly observed for *A. oryzae* walls and not at all for *Neurospora* walls, differential centrifugation was used to remove broken glass beads.

Moisture content of cell walls was determined by drying the cell walls at 80° in vacuum in an Abderharden apparatus. Broken glass beads in the wall preparation was determined as follows: Cell walls were ashed at 600° for 2.5 hr in a platinum crucible. Warm 3 N HCl was added and briefly heated to dissolve the ash. Broken glass beads were collected on a filter paper (Toyo Roshi No. 6) and washed with warm water. Filter paper with broken glass beads was placed in a platinum crucible and heated at 600° for 2.5 hr. The difference from the tare weight was regarded as the weight of broken glass. Weight of cell walls was corrected for moisture content and weight of broken glass.

Laminarin. Insoluble laminarin was purchased from the Institute of Seaweed Research, Musselburgh, Scotland. A solution of 50 g of the material dissolved in 1,000 ml of boiling water was filtered through Toyo Roshi filter paper No. 2. Brown insoluble material was removed by this procedure. When cooled, ethanol was added to a final concentration of 70% (v/v) and the mixture was allowed to stand at -30° overnight. The precipitate was collected by centrifugation, dissolved in 400 ml of water, and dialyzed against deionized water at 4° over night. Brown material precipitated inside the dialysis bag. Slightly turbid supernatant was heated to obtain a clear solution. Snow white laminarin was obtained by lyophilization of this solution. This material was readily soluble in water and was used as a substrate to assay β -1 $\rightarrow$ 3-glucanase. For preparing laminarin plates for the detection of β -1 $\rightarrow$ 3-glucanase activity in chromatographic fractions, 1 g of crude laminarin was dissolved in 100 ml of 0.1 M succinate buffer (pH 5.8) by heating. The insoluble brown material was removed by filtration through Toyo Roshi filter paper No. 2. Agar (2% w/v) and 0.01% Merthiolate were added to the filtrate. After 15 ml portions of the mixture were poured into petri dishes (9 cm in diameter) and the plates were allowed to stand at room temperature until the medium became opaque by the retrogradation of laminarin. Holes (6 mm in diameter) were made with a cork borer and 0.02 ml of enzyme was added to each hole. Extent of lysis measured was after incubation at 38° for 24 hr.

Colloidal pachyman. The sclerotia of a basidiomycete, *Poria* (*Pachyma*) *cocos*, contain up to 93% of a β -1 $\rightarrow$ 3-glucan, pachyman (9). Powder of the sclerotia of this fungus is sold in Japanese drug stores by the name of "Bukuryo." Bukuryo powder was extracted with ether for 20 hr in a Soxhlet apparatus, dried, and dissolved in 10% NaOH solution. The undissolved material was removed by filtration through glass wool. When 10% acetic acid was added dropwise to the filtrate, a gelatinous precipitate of crude pachyman was obtained. The precipitate was again dissolved in 10% NaOH solution, filtered through Toyo Roshi filter paper No. 2, and pachyman was precipitated by the addition of 10% acetic acid solution. This procedure was repeated three times. The white precipitate was dialyzed against deionized water, collected by centrifugation, and homogenized in a homogenizer (Toyo Seiki Co.,

Tokyo) for 3 min. For the detection of β -1 $\rightarrow$ 3-glucanase activity in chromatographic fractions, turbidity of the suspension was made to 0.3 at 660 m μ with 0.02 M phosphate buffer, pH 6.5, (optimum pH for pachyman was 6.5 instead of 5.8). A 15 ml quantity of this colloidal pachyman suspension with 2% agar was poured into a petri dishes (9 cm in diameter). Activity of β -1 $\rightarrow$ 3-glucanase was detected in the same way as was done with laminarin plate. Pachyman was found to be more convenient and economical than laminarin for preparing plates for the detection of β -1 $\rightarrow$ 3-glucanase.

Colloidal chitin. Chitin was separated from crab shell by the method described by REYNOLDS (10). Chitin was ground to a fine powder in a ball mill and sieved through a No. 1.0 sieve. The chitin powder was immersed in 42% NaOH solution and allowed to swell for 3 hr in vacuum at 30°. Alkali-swollen chitin was collected by filtration on a Büchner's funnel. The alkali chitin cake was transferred to a mortar, 3.5 times by weight of crushed ice was added, and chitin cake was kneaded with ice by the use of a pestle. A very viscous, uniformly dispersed colloidal chitin was obtained. This material was dialyzed against distilled water until the suspension became nearly neutral. Chitin was collected by centrifugation and suspended in 0.1 M phosphate buffer (pH 6.5), and again dispersed with the aid of sonic treatment. Chitin plate was prepared in a similar manner as laminarin plate was made.

Colloidal chitin prepared with the use of conc HCl (we refer to this material as acid colloidal chitin in this paper to distinguish it from the colloidal chitin described above) was made according to the method described by BERGER and REYNOLDS (11).

Glycol chitin. Water-soluble glycol chitin was prepared according to the method of SENJU and OKIMATSU (12) from crab shell chitin and ethylene oxide. Glycol chitin was lyophilized.

β -1 $\rightarrow$ 6-Glucans. Zeagallan, a polysaccharide produced by a species of *Ustilago*, corn rust, with about 86% of β -1 $\rightarrow$ 6 linkage and some β -1 $\rightarrow$ 3 bonds, was kindly provided by Mr. MITSUO HIURA of Myojo College, Tokyo.

Luteose, a β -1 $\rightarrow$ 6-glucan, prepared from the broth of *Penicillium aculeatum* RAPER et FENNELL var. *apiculatum* ABE strain No. 2282 (13) was obtained through the courtesy of Mr. NORIO NAKAMURA of Shimodaminami High School of Shizuoka Prefecture. The original strain of this *Penicillium* sp. was no longer available. The authors succeeded in finding a strain of the same species which produces high amount of luteic acid in the broth and most of the assay of β -1 $\rightarrow$ 6-glucanase was made with the luteose obtained from this strain. Detail of the production and characterization of luteose produced by this strain will be described in a separate paper.

Gel filtration. Sephadex G-200 was purchased from the Pharmacia, Uppsala. The gel was allowed to swell in a buffer for 3 days and fine particles were removed by decantation before packing.

Ion-exchange chromatography. Diethylaminoethyl-cellulose (DEAE cellulose) was obtained from the Brown Co., U.S.A. It was treated with alkali,

acid, and alkali in the usual manner and, after thorough washing with water, it was equilibrated with 0.005 M tris (hydroxymethyl) aminomethane (Tris) HCl buffer of pH 8.0.

Analytical methods. Liberation of aldehyde groups during hydrolysis of polysaccharides by enzymes was determined with 3,5-dinitrosalicylic acid reagent according to the method of SUMNER and SOMERS (14). N-Acetylhexosamine was determined with the dimethylaminobenzaldehyde reagent according to the method of REISSIG *et al.* (15).

β -Glucanase assays. Routine assay of β -1 $\rightarrow$ 3-glucanase was made as follows. A solution of 6 mg/ml of laminarin dissolved in 0.1 M succinate buffer at pH 5.8 and enzyme solutions were equilibrated at 38° for 5 min prior to the enzyme reaction. To 0.5 ml of the laminarin solution, 0.5 ml of the appropriate dilution of the enzyme was added. After 5 min, 1 ml of dinitrosalicylic acid reagent was added to stop the enzyme reaction. Reducing groups in the reaction mixture was determined according to the method of SUMNER and SOMERS (14). One unit of β -1 $\rightarrow$ 3-glucanase is defined as that amount of enzyme which produces reducing groups equivalent to 100 μ g of glucose in 1 hr at 38° at pH 5.8. Activity of β -1 $\rightarrow$ 6-glucanase was determined in a similar way except that luteose dissolved in 0.1 M succinate buffer (pH 5.5) was used as a substrate and reaction time was varied according to the activity of the enzyme.

Chitinase assay. Substrate solution: Lyophilized glycol chitin was dissolved in deionized water with the aid of Sono Cleaner (No. 2321 Kaijo Denki Co., Tokyo). Concentration of the glycol chitin solution was adjusted so that the relative viscosity of the solution when added to an equal volume of 0.1 M phosphate buffer (pH 6.5) would be approximately 2. Small amount of insoluble material was removed by filtration through Toyo Roshi filter paper No. 2.

Procedure: Five ml of glycol chitin solution and 4 ml of 0.1 M phosphate buffer (pH 6.5) were pipetted into an Ostwald capillary viscometer of 10-ml capacity. One ml of properly diluted enzyme solution was added. Initial flow time was obtained by substituting a buffer for the enzyme. Readings were taken at short intervals and the time for 40% reduction of viscosity increment was noted (the viscosity increment here is defined as the original relative viscosity from which the relative viscosity of buffer was subtracted). Fig. 3b shows the linear relationship between enzyme concentration and the reciprocal of the time required to reduce 40% of viscosity increment. Ten units of enzyme were defined as that amount which reduces 40% of the viscosity increment of glycol chitin solution in 1 min.

Digestion of the cell walls of fungi. Digestion of fungal cell walls by enzymes was carried out in 100-ml L-shaped tube designed to fit Monod thermo shaking incubator (Otake Work, Tokyo). The cell walls were evenly suspended by sonic treatment (Sono Cleaner, Kaijo Denki, Co., Ltd., Tokyo). Turbidity of the cell wall suspension was measured in an photoelectric color-

Fig. 3a. Reduction of relative viscosity of glycol chitin solution by the action of various concentration of chitinase.

Fig. 3b. Relationship between the enzyme concentration and reciprocal of time to reduce 40% of the viscosity increment of glycol chitin solution.

meter (Hitachi, Model 101, Hitachi Ltd., Tokyo) at 660 $m\mu$. Aliquots were sampled at certain time intervals and the amount of reducing sugars and N-acetylhexosamine was determined as described in analytical methods.

Paper chromatography. For the analysis of mono- and oligo-saccharides in the reaction mixture, paper chromatography with Toyo Roshi, paper No. 54, was carried out. Descending chromatography with a solvent mixture of ethyl acetate-acetic acid-water (3:1:3) and ascending chromatography with a solvent mixture of butanol-pyridine-water (6:4:3) with three multideliverments were most frequently used. Reducing sugars were detected by spraying aniline hydrogen phthalate reagent, and N-acetylhexosamine and its oligomers were detected by the modified Elson-Morgan spray reagent (16).

Electron microscopy. Cell walls with or without enzyme treatment were examined in Superscope (Japan Electron Optics Laboratory Co., Ltd., Tokyo). Cell walls were shadowed with nickel at an angle of 30° prior to examination.

RESULTS

Floating characteristic of the cell walls of Piricularia oryzae P₂

As described in materials and methods, cell walls of *P. oryzae* P₂, when prepared by disrupting the mycelia in Tween 80-EDTA-buffer and washed repeatedly with deionized water, the cell walls tended to remain suspended for indefinite period of time in water when the wall suspension was poured in a glass cylinder and left in an ordinary gravitational field. When several milliliters of saturated CaCl₂ solution was added to 1,000 ml of this wall suspension, cell walls aggregated, showed a kind of flocculent appearance, and then settled within a few minutes. Cell walls also flocculated and settled by the addition of NaCl solution but the effect was not so dramatic as in the case of the addition of CaCl₂ solution. The same procedure of the preparation of the cell walls of *P. oryzae* except for the use of Tween 80-buffer without EDTA at the time of mycelial disruption was followed. The cell walls thus prepared did not acquire the floating characteristic even by repeated washing with deionized water.

Walls of *Aspergillus oryzae* and *Neurospora crassa* were prepared in the same way with Tween 80-EDTA-buffer. Walls of *A. oryzae* partly acquired this characteristic. Walls of *Neurospora crassa*, however, did not gain this characteristic.

Reasonable explanation for this phenomenon has not yet been obtained. Possible causes will be discussed in the discussion section.

Lysis of P. oryzae P₂ walls by Bacillus circulans WL 12

B. circulans WL 12 was inoculated into a medium containing 2% (wet weight/v) *P. oryzae* mycelium and 20% (v/v) laboratory-prepared yeast autolysate in 0.1 M phosphate buffer (pH 7.0). Mycelium disappeared almost completely after 48 hr when the bacteria were grown on shaker at 28°. Cells and debris were removed by centrifugation. The ability of the culture broth to lyse cell walls of *P. oryzae* was tested both by cup plate and turbidimetric methods. A clear lysed zone was obtained on the cell wall agar plate. When *P. oryzae* cell walls were suspended in the culture fluid a rapid lysis occurred.

Specificity of the crude lytic enzyme preparation.

To test polysaccharidase activity of the crude enzyme, the following experiment was carried out. The culture fluid was concentrated 10-fold under vacuum and dialyzed against distilled water. Substrate (2 ml in 0.2 ml of 0.1 M phosphate buffer (pH 6.5) with 0.02% Merthiolate) was dispensed into a test tube. Before the addition of enzyme, 10 μ l of the mixture was spotted on a thin-layer chromatographic Abicel plate as a control. After a certain incubation time at 30°, 20 μ l of the reaction mixture was spotted on an Abicel plate and developed with propanol-ethyl acetate-water (7:1:2). As shown in Table I. the lytic enzyme demonstrated β -1 $\rightarrow$ 3- and β -1 $\rightarrow$ 6-glucanase, and chitinase

Table 1. Specificity of the crude enzyme.

Substrate	Linkage	Hydrolysis
Pachyman	β -1 $\rightarrow$ 3	+
Laminarin	β -1 $\rightarrow$ 3	+
Zeagallan	β -1 $\rightarrow$ 6	+
Luteose	β -1 $\rightarrow$ 6	+
Cellulose (Whatman)	β -1 $\rightarrow$ 4	—
Avicel	β -1 $\rightarrow$ 4	—
Soluble Starch	α -1 $\rightarrow$ 4, α -1 $\rightarrow$ 6	—
Nigeran	α -1 $\rightarrow$ 3, α -1 $\rightarrow$ 4	—
Chitin	β -1 $\rightarrow$ 4 (N-acetylglucosamine)	+
Cellobiose	β -1 $\rightarrow$ 4	—
Laminaribiose	β -1 $\rightarrow$ 3	—
Gentiobiose	β -1 $\rightarrow$ 6	—

activities. Since the substrate was not available, β -1 $\rightarrow$ 2-glucanase was not tested.

Lytic enzyme preparation

As a result of preliminary experiments, the following condition was used for the preparation of lytic enzymes.

Portions of 120 ml of the enzyme production medium containing 2% (wet weight/v) of the mycelium of *P. oryzae* and 20% of laboratory-prepared yeast autolysate in 0.1 M phosphate buffer (pH 7.0) were dispensed in 500-ml Sakaguchi flasks. Fresh culture of *Bacillus circulans* WL 12 grown on AWY-agar slants was inoculated. The bacteria was allowed to grow for 24 hr at 28° on a shaker. One ml of this seed culture was transferred to a Sakaguchi flask containing a fresh enzyme production medium, and the cells were allowed to grow at 28° on a shaker for 72 hr. Cells and debris were removed by centrifugation and the culture fluid was concentrated to about 10% of the original volume in vacuum with a flash evaporator. The concentrated culture fluid was dialyzed against deionized water and then lyophilized.

Fractionation of lytic enzymes

Method for the separation and purification of lytic enzymes has been considerably modified since the time when the experiments described in this paper was conducted. Since details of the separation procedures will be described in a separate paper, only a brief description of the method of separation by which enzymes used in this study will be given here.

The crude lyophilized enzyme was dissolved in 2 ml of 0.1 M phosphate buffer (pH 7.0), centrifuged at 10,000 rpm to remove insoluble material and placed on a column of Sephadex G-200 (2 $\times$ 20 cm) and gel filtration was car-

Fig. 4a. Digestion of the cell walls of *Piricularia oryzae* P₂ by the action of the crude enzyme.

The reaction mixture contained the crude enzyme (480 units/ml of β -1 $\rightarrow$ 3-glucanase, 25 units/ml of β -1 $\rightarrow$ 6-glucanase, and 12 units of chitinase), 1 mg/ml of dry cell walls, and 0.01% Merthiolate in 0.03 M phosphate buffer (pH 7.0). The reaction was carried out at 38° with constant shaking. (—○—) Turbidity at 660 m μ expressed as the percent of the turbidity of the original cell wall suspension. (—△—) Reducing sugars assayed as glucose equivalents and expressed as the percent of the cell wall material. (—●—) N-Acetylhexosamine assayed as N-acetylglucosamine equivalents and expressed as the percent of the cell wall material.

ried out with a flow rate of 70 ml/hr. The large protein peak eluted around void volume of the gel did not have lytic enzyme activities. Activities of β -1 $\rightarrow$ 3-glucanase and chitinase considerably overlapped. Activity of β -1 $\rightarrow$ 6-glucanase appeared in later fractions indicating that this is a small molecular weight protein. Since there was still considerable overlap between β -1 $\rightarrow$ 6-glucanase and other fractions, all the active fractions were combined and dialyzed against 0.005 M Tris-HCl buffer (pH 8.0).

Enzymes treated with Sephadex G-200 were charged on DEAE cellulose column (2 $\times$ 30 cm) and stepwise elution was made with 100 ml of 0.005 M Tris-HCl buffer (pH 8.0), 200 ml each of 0.03 M, 0.09 M, 0.18 M, and 0.3 M of phosphate buffer, and 250 ml of 0.5 M acetate buffer (pH 5.0), at a flow rate of 78 ml/hr.

β -1 $\rightarrow$ 6-Glucanase was eluted at the phosphate buffer concentration of 0.03 M. Chitinase fractions appeared at 0.09 M, and β -1 $\rightarrow$ 3-glucanase was eluted at 0.3 M. There was a possibility of the presence of some other unknown lytic enzyme activity in this " β -1 $\rightarrow$ 6-glucanase" fraction. Contamination test re-

Fig. 4b. Digestion of the cell walls of *Aspergillus oryzae* by the action of the crude enzyme.

Conditions were the same as those described in Fig. 4a.

Fig. 4c. Digestion of the cell walls of *Neurospora crassa* by the action of the crude enzyme.

Conditions were the same as those described in the legend of Fig. 4a.

vealed that this " β -1 $\rightarrow$ 6-glucanase" was free of β -1 $\rightarrow$ 3-glucanase activity. Trace of contaminated chitinase was removed by the following method. β -1 $\rightarrow$ 6-Glucanase fraction was dialyzed against 0.05 M phosphate buffer (pH 7.0) and equal amount of acid colloidal chitin with O.D.₆₆₀ of 0.4 was added. The

Fig. 5. Residual wall material after 7 hr of digestion of *P. oryzae* cell walls with the crude enzyme.

mixture was stirred at room temperature for 5 min, centrifuged at 10,000 rpm, and the supernatant was dialyzed against 0.05 M phosphate buffer (pH 7.0). The β -1 $\rightarrow$ 6-glucanase thus treated was essentially free of chitinase. Chitinase fraction was contaminated with some β -1 $\rightarrow$ 3-glucanase. Therefore chitinase in this study was obtained from other source as described below. β -1 $\rightarrow$ 3-Glucanase fractions did not show either chitinase or β -1 $\rightarrow$ 6-glucanase activity.

Chitinase preparation

For the purpose of obtaining microorganisms which produce a high level of chitinase, a screening was carried out. Among 646 strains of *Streptomyces* spp. isolated from soil, *Streptomyces* CH9 showed the highest activity on the plate. The medium used to screen chitin-decomposing *Streptomyces* was prepared according to REYNOLDS (10). *Streptomyces* CH9 was grown in a medium containing 0.07% K_2HPO_4 , 0.03% KH_2PO_4 , 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0001% $ZnSO_4$, and 0.2% acid colloidal chitin. Before autoclaving, the pH was adjusted to 7.0. *Streptomyces* culture was transferred from agar slant to 500-ml Sakaguchi flask containing 100 ml of the above medium. Cells were allowed to grow at 28° for 7 days. Culture broth showed strong

Fig. 6. Digestion of the cell walls of *P. oryzae* P₂ by the action of β -1 $\rightarrow$ 3-glucanase.

The reaction mixture contained 5 units/ml of β -1 $\rightarrow$ 3-glucanase, 1 mg/ml of dry cell wall, and 0.01% Merthiolate in 0.03 M phosphate buffer (pH 7.0). Reaction was carried out at 38° with constant shaking. (—○—) Turbidity at 660 μ expressed as the percent of the turbidity of the original cell wall suspension. (—△—) Reducing sugars assayed as glucose equivalents and expressed as the percent of the cell wall material.

chitinase activity but neither β -1 $\rightarrow$ 3- nor β -1 $\rightarrow$ 6-glucanase activity was detected. After removal of the cells and debris from the culture broth, chitinase was precipitated at 80% saturation of ammonium sulfate. The enzyme was dissolved in 0.1 M phosphate buffer (pH 7.0) and dialyzed against 0.01 M phosphate buffer (pH 7.0).

Digestion of fungal walls by the crude enzyme preparation

Cell walls of three species of the fungi tested were rapidly lysed by the crude enzyme preparation (Fig. 4a.b.c.). The initial rate of lysis of *P. oryzae* P₂ walls was considerably higher than those of *Aspergillus oryzae* and *Neurospora crassa* walls, although about the same amount of reducing sugars (ca. 200 μ g/mg) was released from the walls of all the three species in the first 1 hr of incubation. Turbidity of the cell wall suspension of *P. oryzae* P₂ decreased to about 30% of the original value while that of the wall suspensions of *Neurospora crassa* and *Aspergillus oryzae* decreased to 60 and 55%, respectively, in the first hr. The most marked difference was the release of N-acetylhexosamine. Release of N-acetylhexosamine from the walls of *Aspergillus oryzae* was 220 μ g/ml after 20 hr of incubation while that from the walls of *Neurospora crassa* and *Piricularia oryzae* was 36 and 8 μ g/ml, respectively.

Fig. 7. Cell walls of *Piricularia oryzae* P₂ digested by the action of β -1 $\rightarrow$ 3-glucanase.

The cell walls in the reaction mixture after 20 hr of incubation shown in Fig. 6 were shadowed and observed as described in Materials and Methods.

These values partly reflect the relative content of N-acetylhexosamine in the walls (17–19). However, since the values for *Piricularia oryzae* and *Neurospora crassa* are too low, there may be other possibilities such as the difference in the state, either structural or compositional, of chitin-containing portion of the cell walls among these fungi.

Lysis of the cell walls of P. oryzae P₂ by the single and combined action of β -1 $\rightarrow$ 3-glucanase, chitinase, and “ β -1 $\rightarrow$ 6-glucanase”

Each of the separated enzymes demonstrated some reduction of the turbidity of the cell wall-suspension and release of reducing sugars from the cell walls.

A substantial decrease in turbidity occurred with only a small amount of reducing sugar liberation by the action of β -1 $\rightarrow$ 3-glucanase (Fig. 6). Turbidity of the cell wall suspension became 36% of the original value after 20 hr of incubation while concomitant release of reducing sugar was only 10.7% of

Fig. 8. Digestion of the cell walls of *P. oryzae* P₂ by the action of chitinase.

The reaction mixture contained 4 units/ml of chitinase, 1 mg/ml of dry cell walls, and 0.01% Merthiolate in 0.03 M phosphate buffer (pH 7.0). The reaction was carried out at 38° with constant shaking. (—○—) Turbidity at 660 m μ expressed as the percent of the turbidity of the original cell wall suspension. (—△—) Reducing sugars assayed as glucose equivalents and expressed as the percent of the cell wall material.

the cell wall material when expressed as glucose. Laminaribiose and glucose were detected on paper chromatogram. Shadowed preparation of the cell walls after 20 hr of digestion with β -1 $\rightarrow$ 3-glucanase had a very rough surface with granular appearance (Fig. 7) compared with the walls without enzyme treatment (Fig. 2).

Some reduction of turbidity and small amount of reducing sugar liberation occurred with this chitinase preparation obtained from the culture filtrate of *Streptomyces* CH9 (Fig. 8). Turbidity was reduced to 61% of the original value and 9.2% of reducing sugar was liberated. Mainly N, N'-diacetylchitobiose and some N-acetylglucosamine were detected on the paper chromatogram when the paper was sprayed with modified Elson-Morgan reagent. Glucose and some other oligosaccharides were also detected, although in a very small amount, when the paper was sprayed with aniline hydrogen phthalate reagent.

With " β -1 $\rightarrow$ 6-glucanase," a considerable amount of reducing sugars was released from the walls although the concomitant decrease in turbidity was only slight (Fig. 9). Mainly glucose was detected on the paper chromatogram. The amount of reducing sugars liberated during 20 hr of incubation reached 24.8% of the cell wall material. Turbidity became 71% of the original value during this period. Not much change in appearance was noted under a phase-

Fig. 9. Digestion of the cell walls of *P. oryzae* P₂ by the action of “ β -1 $\rightarrow$ 6-glucanase”.

The reaction mixture contained 5 units/ml of β -1 $\rightarrow$ 6-glucanase, 1 mg/ml of dry cell wall, and 0.01% Merthiolate in 0.03 M phosphate buffer (pH 7.0). The reaction was carried out at 38° with constant shaking. (—○—) Turbidity at 660 m μ expressed as the percent of the turbidity of the original cell wall suspension. (—△—) Reducing sugars assayed as glucose equivalents and expressed as the percent of the cell wall material.

contrast microscope. Thickness of the cell wall appeared to have been reduced in general, judging from the shadow of the cell walls with and without treatment with this enzyme fraction. Presence of β -glucosidase or oligase was thought to be possible, but action on laminaribiose, gentiobiose, and *p*-nitrophenyl- β -glucoside was negative. Further purification of this “ β -1 $\rightarrow$ 6-glucanase” fraction is now under way. At this stage it is not certain whether the liberation of reducing sugars was all due to the splitting of β -1 $\rightarrow$ 6-glucosidic linkages.

With the combined action of β -1 $\rightarrow$ 3-glucanase and chitinase, initial rate of turbidity reduction was higher than the sum of the rates with the individual enzymes. Turbidity dropped to 43% of the original value in the first 60 min of incubation. However, the cooperative action of these two enzymes for the lysis of cell walls appears to be not as dramatic as it was for the cell walls of other fungi for which individual enzymes showed only a small amount of digestion (Fig. 10).

With the combined action of β -1 $\rightarrow$ 3-glucanase and “ β -1 $\rightarrow$ 6-glucanase,” turbidity reduction was similar to that with β -1 $\rightarrow$ 3-glucanase alone. Liberation of reducing sugars was roughly the sum of the amounts liberated in the reactions with the individual enzyme systems (Fig. 11).

Fig. 10. Digestion of the cell walls of *P. oryzae* P₂ by the action of β -1 $\rightarrow$ 3-glucanase and chitinase.

The reaction mixture contained 5 units/ml of β -1 $\rightarrow$ 3-glucanase and 4 units/ml of chitinase, 1 mg/ml of dry cell wall, and 0.01% Merthiolate in 0.03 M phosphate buffer (pH 7.0). The reaction was carried out at 38° with constant shaking. (—○—) Turbidity at 660 m μ expressed as the percent of the turbidity of the original cell wall suspension. (—△—) Reducing sugars assayed as glucose equivalents and expressed as the percent of the cell wall material.

With chitinase and " β -1 $\rightarrow$ 6-glucanase," turbidity reduction was somewhat higher than that with chitinase alone and release of reducing sugars was additive of those with individual enzymes (Fig. 12).

DISCUSSION

Lysis of fungal cell walls by a single and combined action of β -1 $\rightarrow$ 3-glucanase and chitinase has been studied by several researchers.

By the action of endo- β -1 $\rightarrow$ 3-glucanase from a strain of *Bacillus circulans* isolated by HORIKOSHI, about 90% of hexose in the wall of *Aspergillus oryzae* was solubilized, yet the turbidity of cell wall suspension decreased only slightly. Single use of chitinase did not cause significant release of N-acetylglucosamine. However, with the combination of these two enzymes, turbidity went down to less than 5% of the initial value. From these results, HORIKOSHI indicated that it is probably chitin which imparts the structural strength to the walls (17). In the walls of *Fusarium solani* when incubated with either β -1 $\rightarrow$ 3-glucanase or chitinase alone, there was an insignificant weight loss and no marked morphological change of walls was observed. Combination of these

Fig. 11. Digestion of the cell walls of *P. oryzae* P₂ by the action of β -1 $\rightarrow$ 3-glucanase and " β -1 $\rightarrow$ 6-glucanase".

The reaction mixture contained 5 units/ml of β -1 $\rightarrow$ 3-glucanase, 5 units/ml of β -1 $\rightarrow$ 6-glucanase, 1 mg/ml of dry cell wall, and 0.01% Merthiolate in 0.03 M phosphate buffer (pH 7.0). The reaction was carried out at 38° with constant shaking. (—○—) Turbidity at 660 m μ expressed as the percent of the turbidity of the original cell wall suspension. (—△—) Reducing sugars assayed as glucose equivalents and expressed as the percent of the cell wall material.

two enzymes caused a solubilization of about 94% of the hyphal wall material. In the case of *Fusarium solani*, in contrast to *Aspergillus oryzae* walls, isolated walls have a structure which is somehow protected from the attack of a single enzyme. SKUJINS *et al.* also suggested in their discussion that the chitin-containing wall core, apparently masked by the glucan and possibly other substances, might be imparting shape and rigidity to the cell of *F. solani et al.* (5).

There are similarities and differences in the mode of lysis of *Piricularia oryzae* P₂ walls when compared with those of cell walls of the two species of fungi described above.

Fairly rapid reduction of turbidity of cell wall suspension by the action of β -1 $\rightarrow$ 3-glucanase alone suggests that β -1 $\rightarrow$ 3-glucan-containing layer is important for holding the structure of hyphal wall. The action of the same β -1 $\rightarrow$ 3-glucanase on the living hyphae also strongly supports this view (20). By the digestion of the living hyphae, the outer layer of the cell wall was either ruptured or completely removed. When the suspending medium was osmotically balanced, globular bodies which we refer to as "spheroplast" emerged. These bodies had inner chitin-containing layer around the bodies.

Fig. 12. Digestion of the cell walls of *P. oryzae* P₂ by the action of chitinase and " β -1 $\rightarrow$ 6-glucanase".

The reaction mixture contained 4 units/ml of chitinase, 5 units/ml of β -1 $\rightarrow$ 6-glucanase, 1 mg/ml of dry cell wall, and 0.01% Merthiolate in 0.03 M phosphate buffer (pH 7.0). The reaction was carried out at 38° with constant shaking. (—○—) Turbidity at 660 m μ expressed as the percent of the turbidity of the original cell wall suspension. (—△—) Reducing sugars assayed as glucose equivalents and expressed as the percent of the cell wall material.

The fact that the cells lost their rigidity by the removal of β -1 $\rightarrow$ 3-glucan-containing layer suggests the importance of that layer for holding the shape of hyphae. The elastic nature of the chitin-like material was also indicated.

Co-operative action of β -1 $\rightarrow$ 3-glucanase and chitinase for the lysis of the cell wall of *P. oryzae* was similar to the effect already reported for a number of fungal species. The fact indicates that each one of the glucan and the chitin-like structures is somehow protected from the action of enzyme by the presence of the other structure, and the enzymes can work better, even in the very early stage of lysis, when the rupture of β -1 $\rightarrow$ 3-glucosidic bonds and bonds between N-acetylglucosamine residues takes place side by side.

With the action of the enzyme fraction which we referred to as " β -1 $\rightarrow$ 6-glucanase," a substantial amount of glucose (24.8%) appeared in the medium. Since the concomitant turbidity decrease was only slight and there was no observable change of hyphae under phase-contrast microscope, the polymer of glucose digested by this enzyme fraction appeared to be not important for holding the structure of the walls. The polymers may exist as fillings between β -1 $\rightarrow$ 3-glucan fibers. The other possibility is that β -1 $\rightarrow$ 6-glucan portions are short side-branches of β -1 $\rightarrow$ 3-glucan. Removal of the substance does not lead to

lysis of the walls. It is not yet certain whether all this glucose was liberated by breakage of β -1 $\rightarrow$ 6-glucosidic linkages.

As described in the results, *P. oryzae* P₂ walls acquired a peculiar characteristic of floating for an indefinite period of time when walls were washed repeatedly with deionized water. Reasonable explanation about this phenomenon has not been obtained. About 20% of protein component of the cell wall was found to be dicarboxylic amino acids (aspartic and glutamic acids) and the protein fraction was found to be in the form of glycoprotein linked to a heteropolysaccharide (21). If this protein is localized in the outer part of the cell wall, it is possible that, in normal circumstance, bivalent cations such as Ca²⁺ and Mg²⁺ form bridges between available carboxyl residues. Treatment with ethylenediaminetetraacetic acid should have removed these bivalent cations. The free carboxyl groups should be negatively charged in deionized water. By the addition of only a few ml of saturated CaCl₂ solution to 1,000 ml of wall suspension, flocculation of cell walls occurred within a few min and cell walls settled. Addition of NaCl solution also caused flocculation and sedimentation of walls but much larger amount was necessary. Therefore the effect is considered to be due to cations, Ca²⁺ or Na⁺, instead of the anion, Cl⁻. It is very difficult to draw conclusion just how the walls gained the floating characteristic and how the cations caused flocculation of the walls. If we assume some elastic nature of the cell wall, slight expansion of the cell wall might occur by the removal of bivalent cation bridges and repulsion between negatively charged carboxyl residues might further contribute to this effect. Hydration of charged portion of the wall might also participate in this phenomenon.

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