Multiple $\beta$-1,3 Glucanases in the Lytic Enzyme Complex of
Bacillus circulans WL 12

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The lytic enzyme complex produced by Bacillus circulans WL 12 upon induction with
the mycelia of Piricularia oryzae P2 was analyzed by large scale polyacrylamide gel electrophoresis. Six $\beta$-1,3 glucanases were separated. Activity profiles were obtained of these
multiple $\beta$-1,3 glucanases against P. oryzae P2 cell walls, Saccharomyces cerevisiae walls,
laminarin, oat glucan, Phytophthora glucan and pachyman.

Bacillus circulans WL 121) produces a strong
cell wall lytic enzyme complex when grown
with the mycelia of Piricularia oryzae P2 as the
sole source of carbon. The lytic enzyme com-
plex was found to have $\beta$-1,3 and $\beta$-1,6 glu-
canase and chitinase activities.2) By the use
of polyacrylamide gel electrophoresis, the $\beta$-1,3
glucanase activity was found to be due to at
least six different enzymes. The present paper
deals with the separation of the multiple $\beta$-1,3
glucanases with large scale polyacrylamide gel
electrophoresis and the activity profiles of these
$\beta$-1,3 glucanases on various polysaccharides
containing $\beta$-1,3 glucosidic linkages.

Multiple component natures of $\beta$-1,3 glu-
canases produced by microorganisms have been
reported by various authors.3~6) We consider
the present finding to be particularly interesting
because this might give a clue to the under-
standing of the results of the cross induction
test reported by Tanaka and Phaff.7)

MATERIALS AND METHODS

Organisms. The mycolytic strain used in this study
was Bacillus circulans WL 12.1) As the sources for
the cell walls, Piricularia oryzae P22) and Saccharomyces
cerevisiae IAM 4307 (obtained from the Institute of
Applied Microbiology, University of Tokyo) were
used.

Cultivation of the organisms and the preparation
of cell walls. Media for stock of B. circulans
WL 12 and P. oryzae P2 and the methods for the
cultivation and the preparation of the cell walls of
P. oryzae P2 have been described.2) S. cerevisiae
was grown in Vogel-N-sucrose medium on a shaker at
28°C for 20 hr and cell walls were prepared in a
similar manner as those from P. oryzae P2.

Enzyme assays. Routine assays of $\beta$-1, 3 and $\beta$-
1,6 glucanases and chitinase were performed as described previously.2)

Cell wall lytic activity was determined as follows.
One milliliter of an enzyme solution was added to
2 ml of 0.05 M phosphate buffer, pH 6.8, containing
0.04% of sodium azide; then, 1 ml of the cell wall
suspension containing 4 mg of the cell walls in 0.05 M
phosphate buffer, pH 6.8, was added. Optical density
at 660 nm of the reaction mixture was determined
periodically in a photoelectric colorimeter (Hitachi,
Model 101, Hitachi Co., Japan). Relative optical
density is calculated with the equation.

\[
\text{Relative optical density} = \frac{OD_t - OD_{res.}}{OD_{10} - OD_{res.}} \times 100
\]

where $OD_t$ is the optical density at the incubation
time of t hr, $OD_{10}$ at the initial, and $OD_{res.}$
at the extended period of time. Relative optical density
was plotted against time. Time when the value
reached 50% was noted (Fig. 1). Figure 2 shows the
linear relationship between enzyme concentration
and the reciprocal of the time required to reduce the
value to 50%. Ten units of the lytic activity is
defined as that amount which reduces 50% of the
relative optical density in 1 hr.

Polyacrylamide gel electrophoresis. Disc gel elec-
rophoresis was conducted with an apparatus similar to
FIG. 1. Effect of the Concentration of Lytic Enzyme (Culture Fluid) on the Rate of Reduction in Relative Optical Density of *P. oryzae* P2 Cell Wall Suspension.

Relative concentration of the enzyme: a, 1.0; b, 1/5; c, 1/10; d, 1/30.

Relative optical density was calculated as described in the text.

FIG. 2. Relation between the Enzyme Concentration and Units of Lytic Activity.

The lytic activity was calculated as described in the text.

FIG. 3. Effect of the Concentration of *P. oryzae* P2 Mycelium on Enzyme Induction.

The lytic activity was calculated as described in the text.

that described by Davis\(^8\) using 7.5% acrylamide at pH 9.4 and 4.0\(^9\) with a current of 3 mA/tube at room temperature. The gels were stained with a solution of 0.25% Coomassie Brilliant Blue R 250 in 12.5% trichloroacetic acid as described by Chrambach *et al.*\(^10\) Location of β-1,3 and β-1,6 glucanases and chitinase was determined by the method of Gabriel and Wang\(^11\) using triphenyl-tetrazolium chloride. For β-1,3 glucanases, the gel was immersed in 0.6% laminarin in 0.1 M succinate buffer, pH 5.8, for 10 to 20 min. For β-1,6 glucanase, 0.6% lutean solution in 0.1 M succinate buffer, pH 5.5, was used with an incubation time of 40 to 60 min. Location of chitinases was determined with glycol chitin solution having a relative viscosity of around 2 in 0.1 M succinate buffer, pH 4.8.

For detailed analyses of the activity profiles of the multiple β-1,3 glucanases, a vertical electrophoretic apparatus equipped with a cooling device similar to that described by Raymond\(^12\) was used.

*Substrates.* Preparation of lutean (lucose), colloidal chitin and glycol chitin have been described.\(^2\) Laminarin was purchased from K and K Laboratories Inc., Plainview, New York, U.S.A. Oat glucan, 3-O-β-cellobiosyl-D-glucose and 3-O-β-cellobiosyl-D-glucose were kindly supplied by Dr. A.S. Perlin of McGill University, Montreal, Canada and by Dr. O. Igarashi of Ochanomizu Women's University, Tokyo, Japan. *Phytophthora* glucan (P-glucan) was prepared according to the method of Bartnicki-Garcia.\(^13\)

**RESULTS**

Effect of the concentration of *P. oryzae* P2 mycelia on the production of the lytic enzymes

Hundred milliliters of Yeast Nitrogen Base medium (Difco) containing from 0.5 to 4.0 g of lyophilized *P. oryzae* P2 mycelia was dispensed in a 500 ml Sakaguchi flask and autoclaved. Cells of *Bacillus circulans* WL 12 grown on AWY slant at 28°C for 2 days were suspended in 8 ml of 0.85% NaCl solution and 2 ml of the suspension was inoculated to each flask. Cells were allowed to grow on a reciprocal shaker at 28°C for 3 days. After centrifugation to remove the cells and debris, the culture...
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FIG. 4. Time Course of the Enzyme Production When Mycelium of P. oryzae P2 was Used as a Carbon Source.

broths were dialyzed for 24 hr against deionized water and the enzyme activities were assayed. As shown in Fig. 3, enzyme activities increased rapidly up to the mycelial concentration of 2% and leveled off.

Time course of enzyme production when P. oryzae P2 mycelia were used as an inducer

According to the result described above, Yeast Nitrogen Base medium with 2% P. oryzae P2 mycelia was used as an enzyme production medium. B. circulans WL 12 was inoculated in the same way as described above. Time course of enzyme production was followed by withdrawing aliquots of samples. After centrifugation and dialysis, the activity of each enzyme was assayed.

As shown in Fig. 4, β-1,3 glucanase activity reached about 200 units/ml after 3 days and leveled off whereas the lytic activity against P. oryzae P2 walls attained its maximum after 4 days (50 units/ml) and gradually decreased afterwards. Activities of β-1,6 glucanase and chitinase also reached their maximum after 4 days and leveled off.

Preparation of the crude enzyme complex

According to the time course experiment, the culture broth was harvested after 4 days. After centrifugation, the culture fluid was concentrated about 5-fold under reduced pressure and was dialyzed against deionized water overnight at 2°C. The precipitate formed during the dialysis was removed by centrifugation and the lytic enzymes were precipitated by salting out with ammonium sulfate at 80% saturation. The precipitate was dissolved in 0.1M succinate buffer, pH 6.0, and dialyzed overnight against deionized water. The crude enzyme complex thus obtained had β-1,3 glucanase, β-1,6 glucanase, chitinase and lytic activities, 90 to 95%, 80 to 85%, 90 to 95%, and 90 to 95%, respectively, of the original culture fluid.

Disc polyacrylamide gel electrophoresis of the crude enzyme complex

The electrophoretic patterns of the crude enzyme complex on disc polyacrylamide gels are shown in Fig. 5. A is the protein pattern on pH 9.4 gel. B and C are the activity stains of β-1,3 glucanases on pH 9.4 and pH 4.0 gels. At least three β-1,3 glucanase bands were discernible on both B and C gels. For chitinase and β-1,6 glucanase, only one band for each enzyme was stained on pH 9.4 gel and the active band was not observed on pH 4.0 gel, probably because of the low stability of these enzymes at this pH.

Large scale polyacrylamide gel electrophoresis

The results obtained with disc polyacrylamide gel electrophoresis revealed that β-1,3
glucanase in the lytic enzyme complex of *B. circulans* WL 12 is of multi-component nature. To obtain more detailed information, large scale polyacrylamide gel electrophoretic apparatus was employed.

The gel had 1.0 cm thickness. The separating gel was formed with a size of 2.4 × 12 cm. The electrophoresis was conducted with 7.5% acrylamide at pH 9.4 with a current of 50 mA for 12 hr. The gel was cut along the direction of the current flow into two pieces each being 1.0 × 1.2 × 12 cm (gel A and gel B). Each gel was cut with a razor blade slicer into pieces of 2 mm in thickness from the cathode side. Enzymes were extracted from each slice with 4 ml of 0.1 M succinate buffer, pH 6.0, in a Teflon homogenizer, and used for the assays of the relative lytic activities against the cell walls of *P. oryzae* P 2 and *S. cerevisiae*. To 1.5 ml of the cell wall suspension containing 2.5 mg of lyophilized cell walls was added 1 ml of the extract. Lysis of the cell walls was performed with gentle shaking for 5 hr at 38°C. Relative activity in this case was the optical density at 660 nm at 5 hr of incubation expressed as a percent of the optical density at the start of the reaction.

Each slice from gel B was extracted with 3 ml of 0.1 M succinate buffer, pH 6.0, and relative activities against various substrates were obtained as follows. For laminarin, oat glucan and *Phytophthora* glucan, 0.2 ml of the extract was added to 0.2 ml of 0.1 M succinate buffer, pH 6.0, containing 0.6% of the substrate. The reaction mixture was allowed to stand at 38°C for 90 min. Reaction was terminated by adding 0.4 ml of dinitrosalicylic reagent14) and the reaction mixture was allowed to proceed for 5 min at 38°C with gentle shaking. The reaction was stopped by immersing in boiling water for 1 min. Insoluble residue was removed by filtration through Toyo filter paper No. 2. To 0.1 ml of the filtrate were added 0.5 ml of water, 0.5 ml of 5% phenol solution and 2.5 ml of 96% sulfuric acid. After 10 min, optical density at 490 nm was measured. For the location of chitinase, 0.2 ml of the extracted fraction was added to a cup for antibiotic assays, placed on an agar medium containing acid colloidal chitin. Diameter and degree of clearing were used to locate chitinase in the fractions after incubation for 24 hr at 38°C. For the relative activity of β-1,6 glucanase, 0.2 ml of each fraction from gel B was added to 0.2 ml of 0.6% lutean in 0.1 M succinate buffer, pH 5.5, and incubated at 38°C for 2 hr. The amount of reducing sugars was measured by using dinitrosalicylic acid reagent and the results were expressed as optical density at 540 nm. Figure 6 shows the activity profile of β-1,3 glucanases (extent of laminarin decomposition). At least six enzymes (or possibly eight enzymes) having the ability to decompose laminarin were separated in the large scale polyacrylamide gel electrophoresis. These multiple β-1,3 glucanases were designated as F-Iα, F-Iβ, F-Iγ, F-II, F-III, and F-IV starting...
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FIG. 7. Profiles of Wall Lytic Activities of the Crude Enzyme Complex Induced by P. oryzae P2 Mycelium in Large Scale Polyacrylamide Gel Electrophoresis. Percent reduction in turbidity is used to express the relative lytic activity. (For experimental details, see the text.)

\[ \text{O--O, P. oryzae P2 walls; \textbullet--\textbullet, S. cerevisiae walls.} \]

In Fig. 7, the activity profiles of the decomposition of P. oryzae P2 cell walls and S. cerevisiae are presented. F-II showed the highest $\beta-1,3$ glucanase activity as seen in Fig. 6, whereas the decomposition of P. oryzae P2 cell walls was extensive only with the fractions located in F-I region, and among them, F-Ia was the most active. Cell walls of S. cerevisiae were actively lysed by the enzymes in F-I region. However, it is not certain whether F-Ia was the most lytic fraction as in the case of P. oryzae P3 walls. Two peaks of chitinases were separated by this electrophoresis. No activity of $\beta-1,6$ glucanase was detected with the test condition described above probably due to the inactivation during the procedure.

Figure 8 shows the activity profiles against oat glucan, Phytophthora glucan and pachymann. Decomposition of oat glucan was by far the most extensive with the enzymes in F-I region compared with other $\beta-1,3$ glucanases. Activity of F-III toward oat glucan was very low. Phytophthora glucan was best degraded by F-II. Pachymann was hydrolyzed to some extent by any of the six enzymes. Two small peaks which migrated slightly further than F-IV were active toward pachymann.

DISCUSSION

Since the earlier findings that $\beta-1,3$ glucanase and chitinase causes lysis of Aspergillus oryzae cell walls\(^{15}\) and that $\beta-1,3$ and $\beta-1,6$ glucanases decompose walls of many species of yeasts,\(^{1}\) many publications have appeared concerning the lytic enzymes against eucaryotic microorganisms. These studies have revealed that fungal and yeast cell walls are degraded by single or joint actions of $\beta-1,3$, $\beta-1,6$, $\beta-1,4$ glucanases, chitinase and proteases depending upon the nature of carbohydrates and other wall structures giving rigidity to the cell walls. However, enzymatic decomposition of the cell walls are not as straightforward as it was first expected.

Bacon et al.\(^{5}\) reported that a strain of Cytophaga johnsonii produced several glucanases upon induction with baker's yeast cell walls. One of them that was highly lytic toward thiol treated yeast cell walls appeared to act upon only long chain $\beta-1,3$ glucans producing oligosaccharide with degree of polymerization greater than five. The other $\beta-1,3$ glucanase
producing glucose, laminaribiose and laminartiopiose from laminarin was non-lytic.

Doi et al.\(^6\) separated three \(\beta-1,3\) glucanase from the culture broth of a strain of *Arthrobacter*. Two fractions were active against baker's yeast glucan. Glucanase I liberated an oligosaccharide tentatively identified as laminaripentaose as the major product from the yeast glucan. On the other hand, glucanase II partially lysed yeast glucan and released glucose and laminaribiose. The same authors\(^6\) recently reported that glucanase I was further separated into several components.

Kitamura and Yamamoto\(^17\) also purified a similar enzyme from the culture fluid of a strain of *Arthrobacter luteus* and named it zymolyase.

Yamamoto et al.\(^18\) have crystallized \(\beta-1,3\) glucanases produced by *Rhizopus* sp. and by a species of Fungi Imperfecti. These \(\beta-1,3\) glucanases appeared to have especially high affinities to yeast glucan.

From these results it is evident that not all endo-\(\beta-1,3\) glucanases lyse baker's yeast cell walls effectively and that certain glucanases are highly active. Complexity of the lysis of yeast cell walls was suggested by Tanaka and Phaff\(^7\) by the experiment with a technique they called "cross induction test." *B. circulans* WL 12 was inoculated in a straight line on a rectangular agar block containing the walls of species A (the inducer). Several small square blocks containing the walls of species B,C,D etc. were pushed against the rectangular block. Since *B. circulans* WL 12 does not have colony motility, growth of the bacterium was confined in the center of the block. The set of enzymes induced in response to the inducer A diffused into adjacent blocks and produced clear zones. Although the cell walls used as inducers were almost completely decomposed for the six species of yeasts tested, the lysis of the cell walls of different species of yeast in the square blocks varied considerably according to the combination of the yeast species. The results suggested strongly that there are some significant differences in the structure of yeast glucans between species and that the induction of *B. circulans* WL 12 was complex. This versatile nature of induction is not easily explainable.

The discovery presented in this paper that *B. circulans* WL 12 upon induction with *P. oryzae* P\(_2\) mycelium produces six or more \(\beta-1,3\) glucanase might give a clue to the understanding of this problem. As shown in RESULTS, affinities of the multiple \(\beta-1,3\) glucanases to various \(\beta-1,3\) glucans are quite different from each other indicating that the mechanisms of the hydrolysis might be different. Type of mixed linkages in the glucan (e.g., distribution or arrangement of \(\beta-1,3\) and \(\beta-1,6\) linkages) might cause the differences in the affinities.

To obtain activity profiles of the \(\beta-1,3\) glucanases as close to the native state as possible, no fractionation procedure except ammonium sulfate precipitation was performed prior to the polyacrylamide gel electrophoresis. Two chitinase peaks were detected in the F-I region near F-I\(_a\) and F-I\(_e\) and, therefore, there must have been some synergic actions between the coexisting enzymes for the lysis of *P. oryzae* P\(_2\) walls. However, chitinase activity was not detectable in the region of F-I\(_a\) which showed the highest lytic activity against *P. oryzae* P\(_1\) cell walls. F-I\(_a\) was later purified to the state of homogeneity to give a single protein band in disc gel electrophoresis. The purified F-I\(_a\) without any detectable chitinase activity was also highly lytic toward *P. oryzae* P\(_2\) walls as will be described in a separate paper.\(^19\) It is of interest that the three fractions in the F-I region which were highly lytic toward *P. oryzae* P\(_2\) walls were also very active against oat glucan which has mixed linkages of \(\beta-1,3\) and \(\beta-1,4\).

Purification of each enzyme and detailed study of the action patterns of \(\beta\)-glucans may give fruitful information as to the lysis of cell walls and wall structures of yeasts and fungi.

**REFERENCES**

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