Purification and Properties of F-Ia, a $\beta$-1,3 Glucanase Which is Highly Lytic toward the Cell Walls of 
*Pilicularia oryzae* P$_2$

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F-Ia, which showed the highest lytic activity toward *Pilicularia oryzae* P$_2$ cell walls among the six multiple $\beta$-1,3 glucanases produced by *Bacillus circulans* WL 12 was purified and some of its properties were studied. The preparation was homogeneous by disc polyacrylamide gel electrophoresis at pH 9.4 and 4.0. The molecular weight was estimated to be around 48,000. F-Ia hydrolyzed laminarin by a random mechanism. The enzyme liberated glucose, oligosaccharides and a high molecular weight heteroglycan from *Pilicularia oryzae* P$_2$ cell walls. Heteroglycan showed a single sedimentation peak in ultracentrifugation and contained mannose, glucose and galactose.

In the preceding papers$^{1,2}$ we described that *Bacillus circulans* WL 12 produced multiple $\beta$-1,3 glucanases when grown on the cell walls of *Pilicularia oryzae* P$_2$, *Saccharomyces cerevisiae* and pachyman. Among the multiple $\beta$-1,3 glucanases, F-Ia was the most actively lytic toward *P. oryzae* P$_2$ walls. F-Ia was produced in fair quantity only upon the induction of *P. oryzae* P$_2$ mycelia among the three inducers tested. In this paper, F-Ia was purified to the state of homogeneity to give a single protein band in disc polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

*Enzyme production.* The lytic enzyme complex was induced by *Bacillus circulans* WL 12 with *P. oryzae* P$_2$ mycelia as described previously. After centrifugation and dialysis of the culture fluid, the enzyme complex was precipitated by 80% saturation of ammonium sulfate, dialyzed against deionized water and was used as the crude enzyme preparation.

*Thin-layer chromatography of mono- and oligosaccharides.* For the analyses of mono- and oligosaccharides, thin-layer chromatography with Avicel (Asahi Pharmaceutical Co., Tokyo, Japan) or Wako G (Wako Pharmaceutical Co., Tokyo, Japan) was used. The solvent systems were ethyl acetate, pyridine, water (10: 4: 3) or n-butanol, pyridine, water (70: 15: 10). Sugars were detected with alkaline silver nitrate spray.

*Gas liquid chromatography of sugars.* For the identification and determination of monosaccharides, gas liquid chromatography was performed. After hydrolysis of polysaccharide with 2N sulfuric acid for 4 hr in a boiling bath, the sample was passed through columns of Dowex 50 (acetate type) and Dowex I. The neutral monosaccharides were subjected to trimethylsilylation using a method similar to that described by Sweeley et al.$^3$ A gas chromatograph (JGC 20 K, Japan Electron Optics Laboratory Co., Japan) equipped with a hydrogen flame ionization detector was used. A stainless steel column (3 x 1000 mm) was packed with 3 % OV-17 on Shimalite W (80 ~ 100 mesh). The column temperature was programmed from 140 to 260°C at a rate of 5°C increment per min.

*Infrared absorption spectrum.* Infrared absorption spectrum of F-A (a heteroglycan) was taken by KBr disc method in an infrared spectrophotometer (DF-402G, Japan Spectroscopic Co., Ltd., Japan).

*Molecular weight determination.* Molecular weight of protein was determined by Hayashi and Ohba’s modification$^4$ of Weber and Osborn’s procedure using sodium dodecyl sulfate gel electrophoresis. Molecular markers were purchased from Mann Research Laboratory, Japan.
RESULTS

The crude enzyme complex precipitated by 80 % saturation of ammonium sulfate was dialyzed against deionized water for 24 hr and subjected to large scale polyacrylamide gel electrophoresis. The condition of electrophoresis was the same as described previously except the size of the separating gel which was $1.0 \times 14 \times 12$ cm. After electrophoresis, a rectangular piece of gel $1.0 \times 1.2 \times 12$ cm was cut from the center of the gel and the piece was cut into slices of 2 mm thickness with a razor blade slicer starting from the cathode side. Enzymes were extracted from each slice with 3 ml of 0.1 M succinate buffer, pH 6.0. The $\beta$-1,3 glucanase activity was assayed as described previously and the results were expressed by the optical density at 540 nm. After electrophoresis, a gel piece $1.0 \times 0.5 \times 12$ cm was cut and stained with triphenyltetrazolium chloride after incubation in laminarin solution as described before. Figure 1 shows one example of electrophoretic pattern. F-I region was stained by the activity stain as a broad single band. The region was found to consist of three $\beta$-1,3 glucanase peaks (F-Ia, F-Ib and F-Ic) by slicing and extraction. The region was cut and enzymes were extracted with 0.1 M succinate buffer, pH 6.0. Second electrophoresis of the extracted F-I was performed with the large scale polyacrylamide gel electrophoretic apparatus with a current of 70 mA for 12 hr. Three rectangular pieces $1.0 \times 0.5 \times 12$ cm in size were cut from the middle and two margins and stained with Coomasie Brilliant

Fig. 1. Activity Profiles of $\beta$-1,3 Glucanases of P. oryzae P2 Mycelium Induced Crude Enzyme Complex in Large Scale Polyacrylamide Gel Electrophoresis.

Relative $\beta$-1,3 glucanase (○○○) and lytic (●●●) activities were obtained with the method described previously. Activity stain with triphenyltetrazolium chloride with laminarin as a substrate was done with the method described previously.

Fig. 2. Activity Profiles of $\beta$-1,3 Glucanases and Lytic Activity of F-I on the Second Run Polyacrylamide Gel. ○○○, Relative $\beta$-1,3 glucanase activity; ●●●, relative lytic activity.

Fig. 3. Electrophoretic Patterns of F-Ia on Disc Polyacrylamide Gels.
Blue (Fig. 2). The protein corresponding to F-Iₐ was extracted with 0.1 M succinate buffer, pH 6.0, dialyzed against deionized water and concentrated with a collodion bag. Figure 3 shows the disc electrophoretic patterns of the purified F-Iₐ. Only one protein band was present in the disc gel electrophoretic runs at pH 9.4 and 4.0.

**Optimum pH of F-Iₐ with laminarin as the substrate**

Relative activity of F-Iₐ at different pH values was determined. As shown in Fig. 4, optimum pH of F-Iₐ was around 6.0 when laminarin was used as the substrate although there were some discrepancies in the activity values according to the buffers used.

**Effect of the pH on the stability of F-Iₐ**

The purified F-Iₐ was allowed to stand at 2°C for 24 hr at different pH values. After the treatment, residual β-1,3 glucanase activity was determined by the routine method. As shown in Fig. 5, F-Iₐ was stable in a pH range from 5.0 to 7.0.

**Effect of temperature on the stability of F-Iₐ**

F-Iₐ in 0.1 M succinate buffer was allowed to stand at temperatures between 20°C and 60°C and the residual activity was assayed. Figure 6 shows the results.

**Molecular weight of F-Iₐ**

Figure 7 shows the results of the molecular weight determination by SDS disc gel electrophoresis.
TABLE I. CHROMATOGRAPHIC ANALYSIS OF THE PRODUCTS OF HYDROLYSIS OF LAMINARIN BY F-1a

Reaction mixture contained F-1a and 0.1% laminarin in 0.01M succinate buffer, pH 6.0. The reaction was allowed to proceed at 38°C. Sugars were analyzed by thin-layer chromatography with Avicel with a solvent system of ethyl acetate, pyridine and water (10: 4: 3).

<table>
<thead>
<tr>
<th>Product</th>
<th>Time of incubation (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Glucose</td>
<td>trace</td>
</tr>
<tr>
<td>Laminaribiose</td>
<td>trace</td>
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<tr>
<td>Laminaritriose</td>
<td>+</td>
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<tr>
<td>Laminaritetraose</td>
<td>+</td>
</tr>
</tbody>
</table>

The molecular weight was estimated to be around 48,000 by this method.

**Action pattern of F-1a on laminarin**

Formation of reaction products during the hydrolysis of laminarin was followed with thin-layer chromatography. Table I shows the results. After short time incubation, only oligosaccharides with degree of polymerization higher than three were detected. The higher oligosaccharides gradually disappeared and glucose and laminaribiose were accumulated. The results indicated random mechanism of hydrolysis of laminarin by this enzyme.

**Action of F-1a on oat glucan**

Parrish and Perlin first reported that a β-1,3 glucanase produced by Rhizopus arrhizus acts on oat glucan and accumulates 3-O-β-cellobiosyl-D-glucose and 3-O-β-cellotriosyl-D-glucose as the major products of hydrolysis indicating that both β-1,3 and β-1,4 glucosidic linkages are split by their enzyme. Tanaka and Phaff also observed a similar type of specificity with the partially purified preparation of β-1,3 glucanase from the culture fluids of B. circulans WL 12 (the same strain used in the present study) grown on bakers' yeast cell walls as a carbon source. Similar type of specificity was found with other β-1,3 glucanases. By thin-layer chromatography F-1a was shown to have a similar type of specificity toward oat glucan. It accumulated 3-O-cellobiosyl-D-glucose and 3-O-cellotriosyl-D-glucose as the major products of hydrolysis and also accumulated small quantities of glucose and laminaribiose.

**Decomposition of the cell walls of P. oryzae P2 by the action of F-1a**

To 9 ml of 0.05M phosphate buffer, pH 6.8, containing 12 mg of the lyophilized cell walls of P. oryzae P2, 3 ml of F-1a having β-1,3 glucanase activity of 180 units was added. The reaction mixture was incubated at 38°C with gentle shaking and reduction in optical density at 660 nm, and increases in total and reducing sugars in the supernatant were determined periodically, as shown in Fig. 8.

**Components solubilized from the cell walls of P. oryzae P2 by the action of F-1a**

![Fig. 8. Decomposition of the Cell Walls of P. oryzae P2 by the Action of F-1a.](image-url)
FIG. 9. Gel Filtration on Sephadex G-25 of the Solubilized Fractions from the Cell Walls of P. oryzae P2 by the Action of F-Ia.

Column size: 2 × 130 cm; flow rate: 10 ml/hr. Each fraction contained 3 ml.

In order to obtain the solubilized components of the cell walls by the action of F-Ia, 200 mg of the cell walls were suspended in 30 ml of 0.05 M phosphate buffer, pH 6.8, containing F-Ia with 200 units of β-1,3 glucanase and 0.02 % sodium azide. After 36 hr, the reaction mixture was filtered through Toyo filter paper No. 2, concentrated under vacuum and applied on a column of Sephadex G-25. Relative amounts of carbohydrates in the fractions were determined by the phenol sulfuric acid method and expressed as optical density at 480 nm. As shown in Fig. 9, one sharp peak at the void volume and several peaks of mono- and oligosaccharides were obtained. The higher molecular weight carbohydrate fraction which appeared in the void volume was further fractionated by gel filtration on Biogel P-100. As shown in Fig. 10, there was one large peak with a small shoulder. The fractions from 30 to 38 indicated in Fig. 10 were pooled and the pooled fraction was referred to as F-A.

FIG. 10. Gel Filtration of F-A on Biogel P-100 Column Chromatography.

Column size: 3 × 90 cm.
Column was eluted with water with a flow rate of 15 ml/hr. Each fraction contained 5 ml.

FIG. 11. Ultracentrifugal Pattern of F-A.

Direction of sedimentation is from right to left. The photographs were taken after 25, 41, 57, 73 and 110 min at 52,640 rpm in the Spinco Model E Ultracentrifuge; the temperature was 5.6°C; sample concentration was 0.7%.
Sedimentation pattern of F-A

Sedimentation of F-A in the analytical centrifuge showed a single moving boundary with $s_{20, w}$ approximately 2.0S under the conditions described in Fig. 11.

Infrared absorption spectrum of F-A

Figure 12 shows the infrared absorption spectrum of F-A which was very similar to that of the peptide heteroglycan isolated from P. oryzae P$_3$ cell walls by Nakajima et al.$^{10}$

Analyses of the monosaccharides in F-A

Monosaccharides in F-A were analyzed by gas liquid chromatography as described in MATERIALS AND METHODS. Mannose glucose and galactose in a molar ratio of 8:2:1 were detected.

DISCUSSION

F-I$_a$ which showed the highest lytic activity against P. oryzae P$_3$ walls among the multiple $\beta$-1,3 glucanases produced by B. circulans WL 12 upon induction with P. oryzae P$_3$ mycelium was purified mainly by the use of large scale polyacrylamide gel electrophoresis.

As far as the action patterns on laminarin and oat glucan are concerned, F-I$_a$ showed similar behavior as the $\beta$-1,3 glucanase preparation partially purified from the culture broth of the same strain grown on the bakers' yeast cell.$^6$ Namely, F-I$_a$ hydrolyzed laminarin by a random mechanism; it cleaved both $\beta$-1,3 and $\beta$-1,4 linkages adjacent to $\beta$-1,3 glucosidic linkages in oat glucan.

The purified F-I$_a$ decomposed P. oryzae P$_3$ walls effectively and released glucose, oligosaccharides and a high-molecular weight heteroglucan.

Nakajima et al.$^{10}$ isolated a heteroglycan from the cell walls of the same strain of Piricularia oryzae P$_2$. The heteroglycan contained mannose glucose and galactose in a ratio of 6:2:1. It showed a single sedimentation peak in ultracentrifugal analysis with $s_{20, w}$ of approximately 6.0S. The relation with a heteroglycan obtained in the present study is the problem of a future study.

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