Preparation and Properties of $\beta$-Glucan Synthase of 
*Pyricularia oryzae P$_2$*

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A particulate enzyme fraction capable of catalyzing the transfer of glucose from UDP-glucose to $\beta$-glucan was prepared from the mycelium and protoplasts of *Pyricularia oryzae P$_2$*. An assay method for the $\beta$-glucan synthase was developed. About 80% of the $\beta$-glucan synthase activity was found in the pellet obtained on centrifugation at 28,000 × g for 30 min. The particulate enzyme preparation showed $\beta$-glucan and glycogen synthase activities, in a ratio of 7 : 3. The optimum temperature and pH of the enzyme were found to be 20°C and 7.0, respectively. The glucan synthase activity increased 5.7-fold in 5 hr after the onset of protoplast regeneration.

$\beta$-glucan is one of the most important structural entities in many fungi and yeasts. Therefore investigation of the biosynthesis of $\beta$-glucan is a crucial step for the elucidation of their morphogenesis.

As Farkas$^1$ pointed out in his review, in spite of the importance and abundance of $\beta$-glucan in the cell walls of yeasts and fungi, relatively little is known about its biosynthesis compared with that of chitin and yeast mannan. After the review was published, Shematek et al.$^2$ prepared a cell free enzyme system that catalyzed the biosynthesis of $\beta$-1,3-glucan from the membrane of *Saccharomyces cerevisiae*. The sugar donor was UDP-glucose, and the reaction required the addition of glycerol, bovine serum albumin and ATP or GTP for maximal activity.

We have prepared a particulate enzyme fraction that catalyzes the transfer of glucose from UDP-glucose to $\beta$-glucan from *P. oryzae* mycelium. We developed an assay method for the $\beta$-glucan synthase activity using UDP-$[^{14}C]$glucose and $\beta$-1,3-glucanase$^3$ of *Bacillus circulans* WL-12. The properties of the enzyme system were investigated.

In the previous paper,$^4$ we reported that during the regeneration of *P. oryzae* protoplasts, chitin synthesis preceded glucan formation, and glucan synthesis occurred very rapidly after a lag of about 3 hr. Therefore, $\beta$-glucan synthase activity during the regeneration of *P. oryzae* protoplasts was followed.

**MATERIALS AND METHODS**

Organisms. *Pyricularia oryzae P$_2$* was originally obtained from the National Institute of Agricultural Sciences, Tsukuba, Japan. *Bacillus circulans* WL 12$^5$ was used for the production of the lytic enzyme complex for the preparation of protoplasts and $\beta$-1,3-glucanase for the $\beta$-glucan synthase activity assay.

Chemicals. UDP-$[^{14}C]$-glucose (specific activity, 270 mCi/mmol) was purchased from the Commissariat AL, Energie Atomique. $\alpha$-Amylase from *Bacillus subtilis* and $\beta$-amylase from sweet potato were obtained from Sigma Chemicals Co. (St. Louis, U.S.A.). Isoamylase was the product of Hayashibara Biochemical laboratories Inc. (Okayama, Japan).

Cultivation of *P. oryzae P$_2$*, and preparation and regeneration of protoplasts. Cultivation of *P. oryzae P$_2$*, and

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the preparation and regeneration of protoplasts were conducted as described previously.6)

**Assays.** Assays for β-1,3- and β-1,6-glucanases were performed as described previously,5) using laminarin and lutean as the respective substrates. Laminarin and lutean were prepared in our laboratory from "Makombu" Laminaria japonica, and the cultural broth of Penicillium aculeatum var. aculeatum IFO 5686 (obtained from the Institute of Fermentation, Osaka, Japan), respectively, according to the methods described by Black et al.7) for laminarin and Nakamura et al.5) for lutean (the methods have been considerably modified). Protein was determined by Lowry's method.5)

**Preparation of β-1,3-glucanase free of amylase and β-1,6-glucanase.** B. circulans WL 12 was grown in YNB medium10) containing 1% P. oryzae P2 mycelium and 1.36% NaH2PO4 at 28°C for 84 hr. After centrifugation to remove cells and debris, the culture fluid was concentrated by flash evaporation and then dialyzed against 0.02m phosphate buffer, pH 7.0, and then lyophilized. The amylase-free enzyme was charged on a column of QAE Sephadex equilibrated with 0.02m acetate buffer, pH 4.9, and the fractions showing β,1,3-glucanase were eluted with the same buffer. Beta-1,3-glucanase activity were pooled, concentrated and dialyzed against 0.02m NaH2PO4 at 28°C for 84 hr. After centrifugation to remove cells and debris, the culture fluid was concentrated by flash evaporation and then dialyzed against 0.02m acetate buffer, pH 4.9, and the resultanat solution was passed through a column of starch celite (4:1) for adsorption of the amylase. The amylase-free enzyme was charged on a column of QAE Sephadex equilibrated with 0.02m acetate buffer, pH 4.9, and then β-1,6-glucanase was eluted with the same buffer. Beta-1,3-glucanase free of amylase and β-1,6-glucanase was eluted with 0.3% NaCl, and the fractions showing β-1,3-glucanase activity were pooled, concentrated and dialyzed against 0.02m phosphate buffer, pH 7.0, and then lyophilized.

**Fractionation of cellular materials of P. oryzae P2 for the preparation of β-glucan synthase.** P. oryzae P2 mycelium was grown in Vogel-N-sucrose medium10) at 30°C for 15 to 18 hr. The mycelium was collected and washed with 0.1m Tris–HCl buffer, pH 7.0, containing 1mM EDTA and 1mM mercaptoethanol. Five g wet weight of the mycelium was disrupted with a Dyno Mill (type KDL; Willy A Bachofen Engineers, Basel 5, Switzerland) in a 80 ml glass container, using glass beads of 0.2 to 0.5 mm in diameter, at low temperature maintained by circulating ice-cooled water. The cellular materials were fractionated into four fractions by centrifugation as follows: (1) precipitate on centrifugation at 3,000×g for 30 min, (2) precipitate from the supernatant of (1) on centrifugation at 28,000×g for 30 min, (3) precipitate from the supernatant of (2) on centrifugation at 105,000×g for 60 min, and (4) supernatant obtained in the preparation of (2). Each precipitate was washed once with 0.1m Tris–HCl buffer, pH 7.0, by centrifugation, and then resuspended in a certain amount of the same buffer, and then the β-glucan synthase activity was determined (Fig. 1).

**Standard assay for β-glucan synthase.** According to the data given under RESULTS, the standard assay was conducted as follows. The incubation mixture contained 5mM UDP-[14C]glucose, (2.22×10^5 dpm/μmol), 5mM GTP, 0.8% bovine serum albumin, 8.7% glycerol, 0.08m Tris–HCl buffer, pH 8.0, and 40μl of enzyme solution in a total volume of 100μl. The enzyme solution (particulate enzyme fraction) was subjected to 45 sec ultrasonic treatment with an Ultrasonic Vibrator (Tomy UR-200P, Tomy Seiko Co. Ltd.) with the suspension being immersed in ice water. The incubation was performed for 30 min at 20°C. The reaction was stopped by heating in boiling water for 3 min. Ethanol was added to a final concentration of 70%. The mixture was allowed to stand at −20°C overnight, and then the precipitate was washed six times with 70% ethanol by centrifugation at 3,000×g for 2 min each. Ethanol was removed from the pellet by heating at 50°C and then the sample was suspended in 100μl of 0.13m phosphate buffer, pH 7.0. The β-Glucan synthesized was calculated from the difference of the radioactive activity between the total counts and the counts of the residue after β-1,3-glucanase treatment. For measuring the total radioactive counts, ethanol was added to a final concentration of 70%. For β-1,3 glucanase treatment, 200μl of the enzyme solution (50 units/200μl) was added followed by incubation at 38°C for 30 min, and then ethanol was added to a final concentration of 70%. Both mixtures were allowed to stand at −20°C overnight, and then the suspensions were filtered through glass fiber filters (Toyo Roshi GC50; Toyo Roshi Co. Ltd., Japan), and the filters were washed six times with 70% ethanol and then three times with ethanol–ethyl ether (3:2). The filters were transferred to scintillation vials, liquid scintillator was added, and then the samples were counted with a scintillation spectrometer (Aloka Model LSC 900; Aloka, Tokyo, Japan). The liquid scintillator had the following composition: toluene, 1 liter; 2,5-diphenyloxazol, 4 g; and 1,4-bis[2-(5-phenyloxazoyl)]benzene, 0.1 g. The radioactive
counts after β-1,3-glucanase treatment were subtracted from the total counts. Incorporation of glucose into β-glucan was calculated (4.5 nmol/1000 dpm) and one unit of enzyme was defined as the amount of enzyme required to catalyze 1 nmol of glucose per hour.

RESULTS

Distribution of β-glucan synthase activity in cellular materials fractionated on centrifugation

After disruption of the P. oryzae P2 mycelium, the cellular materials were fractionated as described under MATERIALS AND METHODS, and the β-glucan synthase activity and protein in each fraction were assayed. As shown in Fig. 2, about 80% of the β-glucan synthase activity was found in the pellet (Particulate enzyme fraction) obtained on centrifugation at 28,000 x g for 30 min. Therefore, all the experiments thereafter were conducted using the particulate enzyme fraction.

Minimum β-1,3-glucanase activity required in the assay system

The radioactivity of the residues after β-1,3-glucanase treatment of samples in the β-glucan synthase assay was measured with varying β-1,3-glucanase activity. As shown in Fig. 3, the radioactivity of the residues after β-1,3-glucanase treatment decreased as the β-1,3-glucanase activity was increased, and then leveled off. When the total incorporation of glucose from UDP-glucose was about 13.5 nmol (3,000 dpm), the minimum activity of β-1,3-glucanase required in the system was 14.7 units/300 μl, and therefore β-1,3-glucanase in great excess of this value was used in further experiments.

Time course of glucose incorporation into β-glucan

Figure 4 shows the time course of glucose incorporation into β-glucan up to 80 min under the conditions given in standard β-glucan synthase assay section. Incorporation of glucose increased linearly up to 30 min and then the curve became convex, therefore the incubation time for the standard assay was set at 30 min.

Amylase treatment

UDP-glucose may serve as a glucose donor for both β-glucan and glycogen biosynthesis. To demonstrate that the residue after the β-1,3-glucanase treatment is actually glycogen, the following experiment was conducted. The sample used to determined the total counts in the standard assay procedure described under MATERIALS AND METHODS was treated, respectively, with β-1,3-glucanase, α-amylase, β-

Fig. 2. Distribution of β-Glucan Synthase Activity in Cellular Materials Fractionated on Centrifugation.
amylase, isoamylase, and a combination of \( \beta \)-amylose and isoamylase. The radioactivity of the supernatant obtained with each amylase treatment was expressed as a percentage of that of the residue after \( \beta \)-glucanase treatment. The combined actions of \( \beta \)-amylose and isoamylase released the same amount of radioactivity as in the case of the residue after \( \beta \)-1,3-glucanase treatment, confirming that the residue is actually a glycogen-like polysaccharide.

**Effects of pH and temperature on the \( \beta \)-glucan synthase activity**

Figure 5 shows the \( \beta \)-glucan synthase activity at various pHs and temperatures. The optimum pH and temperature are at around 7.0 and 20°C, respectively.

**Stability of the enzyme**

As shown in Fig. 6, the enzyme lost about 50% of its activity in 8 hr at 4°C. However, when stored at \(-20^\circ\)C in 0.01 m Tris–HCl
Fungal β-Glucan Synthase

**Table I. Effects of Various Metal Ions and Chemicals on the β-Glucan Synthase Activity**

<table>
<thead>
<tr>
<th>Compounds</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
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<tr>
<td>NaCl</td>
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<tr>
<td>KCl</td>
<td>91</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>102</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>79</td>
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<tr>
<td>CaCl₂·2H₂O</td>
<td>83</td>
</tr>
<tr>
<td>FeCl₂·6H₂O</td>
<td>90</td>
</tr>
<tr>
<td>CoCl₂·6H₂O</td>
<td>71</td>
</tr>
<tr>
<td>NiCl₂·6H₂O</td>
<td>44</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>4</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>12</td>
</tr>
<tr>
<td>HgCl₂</td>
<td>7</td>
</tr>
<tr>
<td>EDTA</td>
<td>113</td>
</tr>
<tr>
<td>SDS</td>
<td>68</td>
</tr>
</tbody>
</table>

Note: The final concentration was 1.0 mM.

Effects of metal ions and some chemicals on the β-glucan synthase activity

A preliminary experiment indicated that, in order to test the effects of compounds on the enzyme activity, it is necessary to remove contaminating EDTA as much as possible. Therefore, the particulate enzyme fraction was washed three times by centrifugation with

**Table II. Effects of Various Nucleotides on the β-Glucan Synthase Activity**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
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</tr>
<tr>
<td>AMP</td>
<td>111</td>
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<tr>
<td>ADP</td>
<td>118</td>
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<tr>
<td>ATP</td>
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<td>GTP</td>
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<td>UMP</td>
<td>97</td>
</tr>
<tr>
<td>UDP</td>
<td>94</td>
</tr>
<tr>
<td>UTP</td>
<td>185</td>
</tr>
<tr>
<td>CTP</td>
<td>158</td>
</tr>
</tbody>
</table>

Note: The final concentration of each nucleotide was 500 μM.

buffer, pH 7.0, containing 50% of glycerol, it lost only 10% of its activity in 24 hr.

**Effects of metal ions and some chemicals on the β-glucan synthase activity**

The experimental procedure was the same as that described under "Standard assay for β-glucan synthase," except that the 5 mM GTP in the assay system was replaced with varying amounts of GTP or ATP. The relative activity is expressed as the incorporation of glucose into β-glucan in 30 min.
inhibited by $\text{Co}^{2+}$, $\text{Ni}^{2+}$, $\text{Zn}^{2+}$, $\text{Cu}^{2+}$, $\text{Hg}^{2+}$ and SDS.

**Effects of various nucleotides on the enzyme activity**

Contaminating EDTA was removed as described above and then the effects of nucleotides on the enzyme activity were investigated. As shown in Table II, the activity was enhanced by GTP, ATP, UTP and CTP.

As shown in Fig. 7, the β-glucan synthase activity increased with increasing concentration of GTP and ATP. The activity increased up to the concentration of 100 μM and then leveled off in both cases. The activity became 2.7 and 2.0 times the control level on the addition of more than 100 μM of GTP and ATP, respectively.

**Effect of the UDP-glucose concentration on the enzyme activity**

Figure 8 shows the effect of UDP-glucose on the enzyme activity. The apparent $K_m$ value for UDP-glucose was 1.2 mM.

**Time course of β-glucan synthase activity during the regeneration of P. oryzae P$_2$ protoplasts**

Protoplasts of *P. oryzae* P$_2$ were allowed to regenerate as described previously. Aliquots of samples were withdrawn at various times and then the regenerating protoplasts were disrupted with a Dyno Mill. The particulate enzyme obtained on centrifugation at 28,000 × g for 30 min was suspended in 0.02 M Tris–HCl buffer, pH 7.0, containing 50% glycerol and stored at −20°C for no more than 20 hr, and then the β-glucan synthase activity was determined. As shown in Fig. 9, after a lag of about 1 hr, the enzyme activity increased rapidly during the next 5 hr of regeneration reaching to 5.7-fold the original activity, and then leveled off.

**DISCUSSION**

In this paper, a novel method for the assaying of β-glucan synthase activity, and the preparation and some properties of the enzyme of *P. oryzae* P$_2$ are described.

Literature concerning fungal cell wall glucan biosynthesis up to 1978 was well reviewed by Farkas. He also discussed in detail the difficulty encountered in isolating a cell free system which catalyzes the biosynthesis of wall glucan in fungi and yeasts. He pointed out several factors that could be expected to negatively influence the isolation and the assaying of fungal glucan synthase.

Wang and Bartnicki-Garcia prepared a mixed membrane fraction which catalyzed the abundant formation of β-glucan microfibrils with UDP-glucose as a precursor, from
mechanically disrupted cells of *Phytophthora cinnamomi*. However, they did not mention much about the enzymatic properties of the enzyme system.

Shematek *et al.*2) prepared a membrane fraction which catalyzed the rather efficient transfer of glucose from UDP-glucose. The reaction required the addition of glycerol, bovine serum albumin and ATP or GTP for the maximal activity.

We also succeeded in preparing a membrane fraction (particulate enzyme preparation), which catalyzed the transfer of glucose from UDP-glucose in a reaction mixture with a similar composition to that Shematek *et al.*2) used, from disrupted cells of a rice blast fungus *Pyricularia oryzae*.

We assumed that the major polysaccharides synthesized in our system with UDP-glucose as an immediate precursor were wall glucan and glycogen. The material obtained after incubation of the reaction mixture containing the particulate enzyme, UDP-[14C]glucose, glycerol, bovine serum albumin and GTP, as described for the standard β-glucan synthase assay procedure was treated with β-1,3 glucanase of *Bacillus circulans* WL 12. The radioactivity of the glucanase treated residue (glycogen) was subtracted from the total radioactivity, and the value obtained was used to calculate the β-glucan synthesized. That the radioactive material obtained after β-1,3-glucanase treatment was actually a glycogen-like polysaccharide was shown by the fact that the radioactivity released from the synthesized material through the combined actions of β-amylase and isoamylase coincided very well with the radioactivity of the residue of the synthesized material after β-1,3 glucanase treatment.

It is very interesting that the Km value for UDP-glucose with our system (1.2 mm) is similar to the value in the case of the system of Shematek *et al.*2) (1.7 mm).

Although the data are not conclusive, gel filtration on Sephacyr S-500 indicated that the molecular weight of the glucan synthesized is over $5 \times 10^5$, and an experiment involving Smith degradation indicated that the glucan contained β-1,6-linkages besides β-1,3-linkages.

The time course of β-glucan synthase activity during regeneration of *P. oryzae* P2 protoplasts was followed. After a lag of about 1 hr, the activity of the enzyme increased rapidly during the next 5 hr of regeneration, and then leveled off. The results coincided well with our previous results4) for glucan biosynthesis by protoplasts on gas liquid chromatography, in that glucan was rapidly synthesized during 3 ~ 5 hr of protoplasts regeneration.

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