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Studies on Fungicides. X.[1] Biosyntheses of β-Glucan and Chitin-like Substance of Cell Wall from *Cochliobolus miyabeanus*. (1)

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The biosyntheses of the main components of mycelial cell walls on *Cochliobolus miyabeanus* (β-glucan, β-1,3-linked glucan having branched units connected through C-6 and C-1; chitin-like substance, β-1,4-linked N-acetylglucosamine having branched units of N-acetylgalactosamine connected through C-1 by α-glycosidic linkage) were studied. The incorporation of ^{14}C-glucose or ^{14}C-N-acetylglucosamine from uridine diphosphate (UDP)-derivative into β-glucan or chitin-like substance was catalyzed by the particulate enzymes obtained from mycelia. The optimum conditions of these synthetic reaction were as follows: for glucan; 0.08M Tris-HCl buffer pH 8.2 (contained 0.01M $MgCl_2$ and 0.001M ethylenediamine tetraacetic acid (EDTA)), cell wall glucan, 0.08M UDP-^{14}C-glucose (5 μCi/ml) and enzyme solution; for chitin-like substance; 0.08M Tris-HCl buffer pH 7.5 (contained 0.01M $MgCl_2$ and 0.001M EDTA), cell wall chitodextrin, 0.08M UDP-^{14}C-N-acetylglucosamine (5 μCi/ml) and enzyme solution.

Introduction

In the previous paper,[3,4] the authors reported the presence of the glucan (β-1,3 linked glucan having branched units connected through C-6 and C-1) and the chitin-like substance (β-1,4 linked 2-acetamide-2-deoxy-D-glucose having branched units of 2-acetamide-2-deoxy-D-galactose connected through C-1 by α-glucosidic linkage) in the cell wall of *Cochliobolus miyabeanus*.

The present paper deals with the biosyntheses of these main components of mycelial cell wall described above. Although many reports have been presented on the cell free system of the cell wall chitin biosynthesis,[5,6] little is known on the β-glucan[7,8] which is one of the main components same as to the chitin-like substance. This research was done as a fundamental experiment in order to clarify the biosynthetic mechanisms of the cell wall components in *Cochliobolus miyabeanus*.

Material and Method

1) **Harvesting of the Fungous Mycelium**——The fungous strain was kindly supplied by Dr. Oku (Okayama University). Three-hundred ml of flasks containing 100 ml of 2% potato sucrose liquid medium were inoculated with a suspension of mycelium obtained from a slop culture of the fungus. After shaking at 27° for 48 hr, the colorless mycelium was harvested by filtration through to Toyo Roshi No. 2 filter paper and washed thoroughly with distilled water, essentialy free from medium components.

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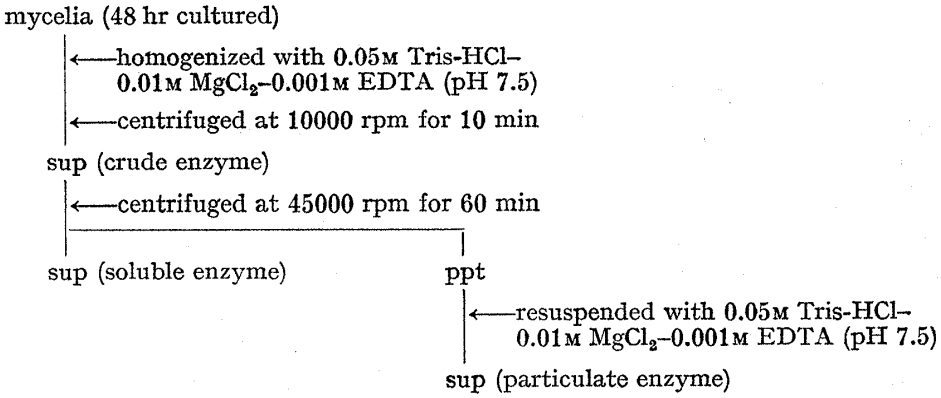

Chart 1. Preparation of Enzyme Solution from Mycelia

2) **Preparation of the Enzyme Solution**——The preparation of the enzyme solution (crude enzyme, particulate enzyme and soluble enzyme) from mycelium was described in Chart 1. The preparation was treated below 4°.

3) **Assay of Glucan Synthetase or Chitin Synthetase**——The reaction systems as shown in Table I were incubated at 25° (for chitin-like substance synthesis) or 27° (for glucan synthesis) for 60 min, followed by adding EtOH to 80% into 1 ml of the reaction systems. After still standing for 12 hr at 4°, the EtOH solution was centrifuged at 3000 rpm for 5 min and the isolated precipitate was dissolved in 1 ml of distilled water. The aqueous solution was deproteinized by shaking with the chloroform–methy alcohol mixture (9:1) for 1 min, and the radio activity of aqueous layer (0.1 ml) was measured by liquid scintillation spectrometer (Fujitsu liquid scintillation counter model EA-118).

Table I. Reaction Systems for Syntheses of Glucan or Chitin-like Substance by Crude Enzyme

For glucan	
0.08 M Tris-HCl buffer pH 8.2	—2.0 ml
(contained 10 mM $MgCl_2$, 1 mM EDTA, 1 mM glucose, 1 mM ATP, 0.01 mM nucleotide)[a]	
cell wall glucan (20 mg/ml)	—0.3 ml
^{14}C-glucose (1.2 μCi/ml)	—0.2 ml
crude enzyme (750 mg/ml protein)	—2.5 ml
(contained hexokinase 37 unit/ml and glucomutase 1.2 mg/ml in 0.01 M phosphate buffer pH 7.1 —0.25 ml)	
For chitin-like substance	
0.08 M Tris-HCl buffer pH 7.53	—2.0 ml
(contained 10 mM $MgCl_2$, 1 mM EDTA, 1 mM ATP, 0.01 mM nucleotide,[a] 1 mM glucosamine)	
cell wall chitodextrin (20 mg/ml)	—0.3 ml
^{14}C-N-acetylglucosamine (1.0 μCi/ml)	—0.2 ml
crude enzyme (750 mg/ml protein)	—2.5 ml
(contained hexokinase 37 unit/ml and glucomutase 1.2 mg/ml in 0.01 M phosphate buffer pH 7.1 —0.25 ml)	

a) A reaction mixture contained one of nucleotides as follows, ATP, UTP, CTP, TTP or GTP.

4) **Assay of the Laminarinase or β-N-Acetylglucosaminidase**——The laminarinase or β-N-acetylglucosaminidase assay involves determination of the released glucose or N-acetylglucosamine from laminarin or chitodextrin by glucostat reagent (Worthington Biochemical Co., New Jersey U.S.A., for glucose) or by the method of Morgan–Elson (for N-acetylglucosamine). The reaction systems (total volume 10 ml; final pH 7.52) was contained crude enzyme (918 mg/ml protein) 5 ml and 100 mg/ml laminarin in 1/10M phosphate buffer (pH 7.52) 5 ml or crude enzyme (620 mg/ml protein) 5 ml and cell wall chitodextrin (54 mg/ml) in 1/20M phosphate buffer (pH 8.2) 5 ml.

5) **Measurement of Radioactivity**——Radioactivity in aqueous solution was determined on 0.1 ml sample, which were added to scintillation vials containing 10 ml of counting fluid of the following composition: 1.6 g of 2,5-diphenyloxazole (PPO), 0.16 g of 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene (dimethyl-POPOP), 40 g of naphthalene: 400 ml of dioxane: toluene: methyl cellosolve mixture (75: 15: 10), and counted with scintillation counter.

Result

1) Incorporation of ^{14}C-Glucose and ^{14}C-N-Acetylglucosamine into Mycelial Cell Walls

In order to prepare the mycelial fragment suspension, the harvested mycelium was homogenized with 1% potato sucrose liquid medium by Warling blender for 10 sec below 4°. After adding ^{14}C-glucose or ^{14}C-N-acetylglucosamine (7 μCi/min) into the mycelial fragment suspension, it was shaken at 27°. The aliquot was withdrawn at interval and growing mycelium was harvested by centrifugation. The cell wall fractions of these mycelia were obtained by the method of described in previous paper,[3,4] and 100 mg of the cell wall was fractionated to β-glucan and chitin-like substance by the way shown in Chart 2. The radio-activities of the each fractions were measured by a liquid scintillation spectrometer, after dissolving in distilled water (β-glucan) or hydrolyzing by 6N HCl (chitin-like substance).

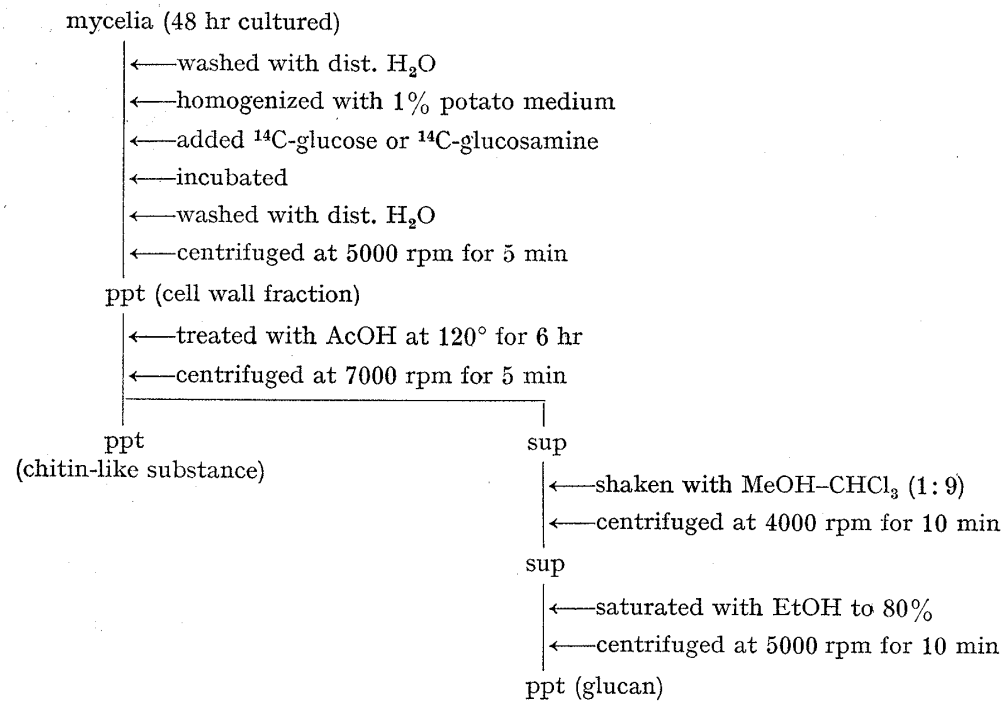

Chart 2. Preparations of Cell Wall, β-Glucan and Chitin-like Substance

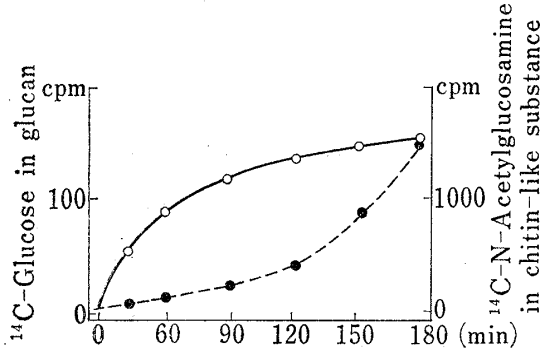

Fig. 1. Incorporation of ^{14}C-labelled Compounds into Cell Wall

—○—: ^{14}C-glucose
---●---: ^{14}C-N-acetylglucosamine

As shown in Fig. 1, while ^{14}C-glucose was incorporated into the cell wall glucan without any lag time, a lag time for 30—60 min was required to initiate incorporation of ^{14}C-N-acetylglucosamine into the cell wall chitin-like substance, and the increasings of radio-activities of both compounds in the cell wall were continued still later of 150 min. These results indicate that mycelial fragments actively synthesize the cell wall by making use of added glucose and N-acetylglucosamine. At first, in order to establish the cell free preparation system which synthesize the cell wall glucan and chitin-like substance, the effects of some nucleotides on syntheses of cell wall components were investigated.

2) The Effect of Some Nucleotides on Incorporation of ^{14}C-Glucose or ^{14}C-N-Acetylglucosamine into the Macromolecular Fraction by Crude Enzyme

Since it is considered that some nucleotide sugars play the role of precursor for polysaccharide syntheses, the following experiments were undertaken to clarify which nucleotide derivative is available for precursor. Two reaction systems as shown in Table I were incubated at 27° or 25° and at interval the aliquots of them were withdrawn, followed by determination of incorporated radio-activites in macromolecular fraction. Table II shows these results.

Table II. Effects of Various Nucleotides on Syntheses of Glucan and Chitin-like Substance by Crude Enzyme

Synthesis of Glucan

Nucleotide	Reaction time (min)			
	30	60	90	120 (cpm)
UTP	3542	6564	8732	10474
GTP	—	25	147	131
CTP	—	59	45	120
ATP	—	121	491	1091
TTP	—	30	49	53

Synthesis of Chitin-like Substance

Nucleotide	Reaction time (min)			
	30	60	90	120 (cpm)
UTP	2011	5827	7433	9112
GTP	—	212	136	109
CTP	—	100	121	150
ATP	—	69	150	381
TTP	—	57	111	159

In the case, when urideine 5′-triphosphat (UTP) was added, the incorporations of ^{14}C-glucose or ^{14}C-N-acetylglucosamine were stimulated most strongly and the adenosine triphosphate (ATP) addition gave 1/10—1/20 (a tence—a twentyth) stimulating effect to the case of UTP addition. No other nucleotides showed any effects. These results suggested that glucose and N-acetylglucosamine are incorporated into the cell wall macromolecular fraction *via* uridine diphosphate (UDP)-derivatives.

3) Incorporation of UDP-^{14}C-Glucose or UDP-^{14}C-N-Acetylglucosamine into the Cell Wall Macromolecular Fraction

Since it was clarified that some UDP-sugars play the role of precursor for cell wal glucan, the condition for incorporation of UDP-^{14}C-sugar into cell wall polysaccharide fraction was studied. As shown in Table III and IV, the radio-activities of polysaccharides were actively increased by adding the particulate enzyme fraction and the cell wall glucan or the cell wall chitin-like substance into the reaction systems. Furthermore, it is an interesting fact that by adding laminarin obtained from *Eisenia bicyclis* or crustacean chitodextrin in stead of the cell wall glucan or the cell wall chitodextrin-like substance, fairly incorporation of the radioactive sugar into the polysaccharide fraction was observed. In the case which added the soluble enzyme fraction as synthetase, few radio-activities of the polysaccharides were increased. The incorporation of radio-active compounds from UDP-^{14}-C-sugars into the polysaccharide fraction by the particulate enzyme under the existence of cell wall glucan or

Table III. Effects of Polysaccharides on Glucan Synthesis by Fractionated Enzymes[a)]

Enzyme	Added polysaccharide	^{14}C-Glucose in glucan (cpm)
Particulate enzyme	cell wall glucan	30839
	laminarin	26204
	H_2O	2774
Soluble enzyme	cell wall glucan	1002

a) Incubation time of reaction mixture was 60 min.

Table IV. Effects of Amino Sugars on Chitodextrin Synthesis by Fractionated Enzymes[a)]

Enzyme	Added amino sugar	^{14}C-GluNAc in chitin-like substance (cpm)
Particulate enzyme	cell wall chitodextrin	30638
	crustacean chitodextrin	21114
	H_2O	9016
Soluble enzyme	cell wall chitodextrin	2900

a) Reaction mixture was incubated for 45 min.
GluNAc: N-acetylglucosamine

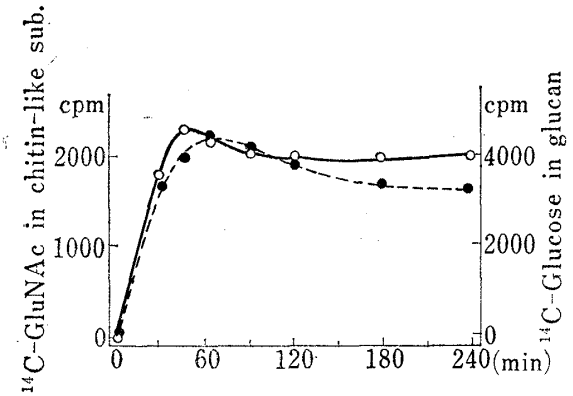

Fig. 2. Incorporations of ^{14}C-Glucose or ^{14}C-N-Acetylglucosamine from UDP-^{14}C-Glucose or UDP-^{14}C-N-Acetylglucosamine into Cell Wall Glucan and Chitin-like Substance

—○—: ^{14}C-glucose
---●---: ^{14}C-N-acetylglucosamine

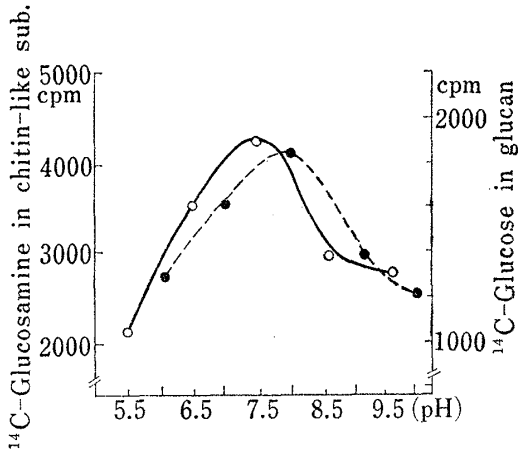

Fig. 3. Effects of pH on Syntheses of Glucan or Chitin-like Substance by Particulate Enzyme

---●---: ^{14}C-glucose
—○—: ^{14}C-glucosamine

chitodextrin-like substance is shown in Fig. 2. The increasing of the radio-activities of glucan fraction was initiated without any lag time and the maximum peak came to after 45 minutes, on the other hand, the incorporation of ^{14}-C-N-acetylglucosamine into the chitodextrin reached to peak after 60 min. The influences of pH and temperature on glucan and chitin-like substance syntheses by particulate enzyme were shown in Fig. 3, 4. From these results, the optimum conditions on polysaccharides syntheses were determined as follows: for glucan, pH 8.2, 27° and for chitin-like substance, pH 7.5, 25°.

4) Hydrolyses of Laminarin or Chitodextrin-like Substance by Mycelial Enzyme

In Fig. 2, a little decrease of radio-activities were observed just after peaks, and these results suggested that synthesized glucan and chitin-like substance were secondarily allowed to react with some hydrolytic enzymes. To make clear these reactions, hydrolyses of laminarin

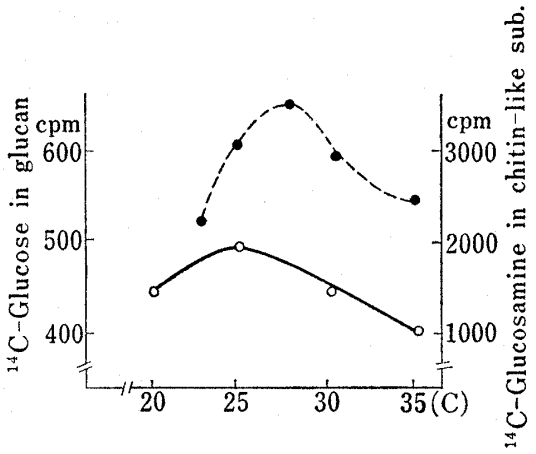

Fig. 4. Effects of Temperature on Syntheses of Glucan or Chitin-like Substance by Particulate Enzyme

---●---: ^{14}C-glucose
—○—: ^{14}C-glucosamine

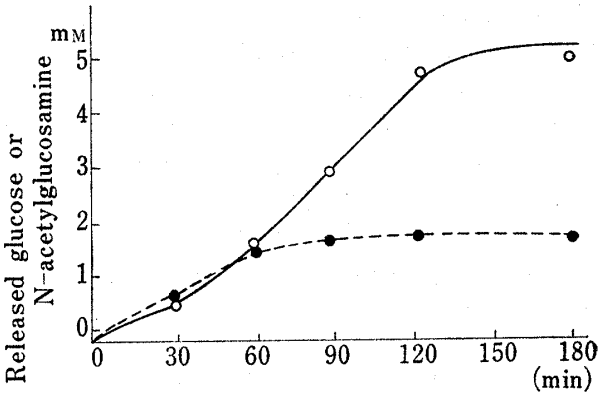

Fig. 5. Hydrolyses of Laminarin or Chitodextrin by Crude Enzyme

—○—: glucose
---●---: N-acetylglucosamine

or chitodextrin-like substance by the crude enzyme were studied. As shown in Fig. 5, fairly amount of glucose or N-acetylglucosamine are released from laminarin or chitodextrin and these results support the suggestion described above.

Discussion

In the previous paper, the authors reported on the fine structures of β-glucan and chitin-like substance which are the cell wall main components in *Cochliobolus miyabeanus*.[3,4] This paper deals with the syntheses of the cell wall glucan and chitin-like substance by cell free system. As shown in Fig. 1, by adding some ^{14}C-glucose to the mycelial fragment suspension, the labelled glucose is rapidly incorporated but in the case of adding some ^{14}C-N-acetylglucosamine, 30 to 60 min lag time is required. Since the incorporation of ^{14}C-N-acetylglucosamine into the chitin-like substance reached the peak more slowly than the incorporation of ^{14}C-glucose into glucan in cell free systems (Fig. 1), it is not considered that the cause of such lag time simply results from the difference of permeabilities between these two labelled compounds. Table II suggests that the added ^{14}C-glucose or ^{14}C-N-acetylglucosamine was incorporated into the glucan or the chitin-like substance through the corresponding UDP-sugar derivatives by the crude enzyme solution. Ozbun, *et al*[9] reported that adenosine diphosphate (ADP)-glucose participated in the polysaccharide synthesis by spinach leaves. Although, in the case of our fungous mycelia, a little stimulating effect was observed by addition of ATP (Table II), it is not clear whether some ATP compete with UTP in the mycelial cells. No stimulating effect was observed by addition of guanine triphosphate (GTP), cytosine triphosphate (CTP), thymine triphosphate (TTP) which participate in the biosyntheses of the plant cell glucan[10,11] and bacterial cell wall polysaccharides.[12–15] Moor[16] reported that UDP-glucose participated in polysaccharides synthesis of yeast. The possibilities of polysaccharide syntheses

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through UDP-sugars by soluble or particulate enzyme fraction obtained from crude enzyme are shown in Table II. Glaster, *et al*[5] found the chitin N-acetylglucosaminyl transferase (chitin synthetase) activity in the precipitation from the mycelial homogenate of *Neurospora crassa* by centrifugation at 140000×***g*** for 60 min and Poter, *et al*[6] reported the presence of the same enzyme activities in the mycelial microsome and mitochondria of *Allomyces macrozyus*. In the case of our fungus, the same mycelial fraction (particulate enzyme) indicated the highest activities of glucan and chitin-like substance syntheses under the following conditions: pH 8.2, 25° (for glucan); pH 7.5, 28° (for chitin-like substance); (Fig. 3, 4), while no activities were shown in the soluble enzyme fraction (Table III, IV). From these results, it is presumed that these enzymes are bound on the membrane as like the enzymes participated in bacterial cell wall syntheses. Mycelial chitin synthetases of *Neurospora*[5] and *Allomyces*[6] are activated by addition of chitodextrin and in the case of our fungus, the syntheses of glucan and chitodextrin are stimulated by addition of glucan or chitodextrin-like substance obtained from our fungal mycelia. Main components of *C. miyabeanus* cell walls are the branched β-glucan and the branched chitin-like substance, however, the laminarin or the crustacean chitodextrin which are the straight chains of β-1,3-glucan or β-1,4-polymer of N-acetylglucosamine indicate the same degree of stimulating effect as mycelial glucan or chitodextrin-like substance, and these results suggest that activating compounds are not always necessary having the same chemical structure as mycelial cell wall components. A study on mechanisms of these activating effects by added compounds are proceeding. Fig. 5 shows some hydrolytic enzymes are contained in the mycelial crude enzyme and under this condition, a part of the synthesized polysaccharide is hydrolysed by these enzymes. The chemical structures of synthesized polysaccharides by mycelial enzymes and the identities of these compounds of cell walls from *Cochliobolus miyabeanus* will appear in a subsequent paper.