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Studies on Fungicides. XII.[1] Relationships between Cell Wall Glycan Synthesis and Hydrolysis by Mycelial Enzymes on *Cochliobolus miyabeanus*

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The relationships between the cell wall glycan synthesis and hydrolysis by mycelial crude enzyme were studied. The crude enzyme indicated some hydrolytic activities to the synthesized β-glucan or chitin-like substance under the optimum conditions for respective glycan synthesis. The most of all enzyme activities were found in the microsomal fraction (glucan synthetase and chitin synthetase) and the soluble fraction (β-glucanase and β-N-acetylglucosaminidase). The activities of the β-glucan synthetase or chitin synthetase contained in microsomal fraction were not affected by addition of uridine diphosphate (UDP)-N-acetylglucosamine (into β-glucan synthetic system) or UDP-glucose (into chitin synthetic system). While the activities of the β-glucanase or β-N-acetylglucosaminidase contained in the soluble fraction were inhibited by addition of UDP-N-acetylglucosamine (into β-glucanase assay system) or UDP-glucose (into β-N-acetylglucosaminidase assay system). Therefore, when the mycelial crude enzyme was used as synthetase, it is evident that UDP-N-acetylglucosamine (precursor of chitin) in the β-glucan synthetic system of UDP-glucose (precursor of β-glucan) in the chitin-like substance synthetic system is not a stimulator of synthetase but an inhibitor of hydrolytic enzymes. The possible role of these phenomena at the time of cell wall growth was discussed.

Introduction

In the previous papers,[3,4] the authors proposed the possible biosynthetic pathway of polysaccharides in cell walls of *Cochliobolus miyabeanus* and just when these polysaccharides were synthesized by crude enzyme, after incorporation maximum of uridine diphosphate (UDP)-^{14}C-sugars, a little decrease of radioactivitiy was observed. This decrease would suggest that synthesized glucan and chitin-like substance were secondarily allowed to react with some hydrolytic enzymes.

In the present experiments, the activity of hydrolytic enzymes in the mycelial crude solution was determined and the relationship between the glucan synthesis and the hydrolytic activity in the crude enzyme was discussed.

Material and Methods

1) **Preparation of Enzyme Solution**——The preparation of the enzyme solution (crude, particulate and soluble enzymes) from mycelia was described in the previous papers.[3,4]

2) **Incorporation of UDP-^{14}C-Glucose and UDP-^{14}C-N-Acetylglucosamine into the Cell Wall Glycan**——The experimental conditions were already described in the previous paper,[3] and the typical reaction systems were shown in Table I.

3) **Assay of β-Glucanase or β-N-Acetylglucosaminidase**——The activity of β-glucanase of β-N-acetylglucosaminidase was assayed under the optimum conditions for cell wall glycan synthesis as described in the previous paper.[3] The enzyme assay involved determination of the released glucose or N-acetylglucosamine from cell wall β-glucan or chitodextrin by glucostat reagent[3] for glucose or by the method of Morgan-Elson[3]

1) This work presented in the part at the Annual Meeting of the Phytopathological Society of Japan, 1974.
2) Location: *Motoyama-Kitamachi, Higashinada-Ku, Kobe.*
3) H. Nanba and H. Kuroda, *Chem. Pharm. Bull.* (Tokyo), **22**, 610 (1974).
4) H. Nanba and H. Kuroda, *Chem. Pharm. Bull.* (Tokyo), **22**, 1895 (1974).

for N-acetylglucosamine. The reaction systems contained crude enzyme (907 mg/ml protein) or soluble enzyme (472 mg/ml protein) 5 ml, cell wall glucan (100 mg/ml) in 0.05 M phosphate buffer pH 7.52 (for β-glucanase assay) or cell wall chitodextrin (54 mg/ml) in 0.1 M phosphate buffer pH 8.2 (for β-N-acetylglucosaminidase assay) 5 ml and UDP-N-acetylglucosamine (10 mg/ml) or UDP-glucose (10 mg/ml) 0.2 ml.

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

For glucan		For chitin-like substance	
0.08 M Tris-HCl buffer pH 8.2 (contained 0.01 M $MgCl_2$, 0.001 M EDTA)	−0.5 ml	0.08 M Tris-HCl buffer pH 7.5 (contained 0.01 M $MgCl_2$, 0.001 M EDTA)	−0.5 ml
cell wall glucan	−0.5 ml	cell wall chitodextrin	−0.5 ml
0.08 M UDP-^{14}C-glucose	−0.5 ml	0.08 M UDP-^{14}C-N-acetylglucosamine	−0.5 ml
enzyme solution	−0.5 ml	enzyme solution	−0.5 ml
with or without 0.08 M UDP-N-acetylglucosamine	−0.1 ml	with or without 0.08 M UDP-glucose	−0.1 ml

Results and Discussion

In the previous papers,[5,6] the authors reported the presence of the β-glucan and the chitin-like substance in the cell walls of *Cochliobolus miyabeanus* as main components and proposed the biosynthetic pathways of these compounds.[3,4] As shown in Chart 1, the mycelial enzymes stimulated incorporation of glucosyl residue or N-acetylglucosaminyl residue into the β-glucan or chitin-like substance from UDP-glucose or UDP-N-acetylglucosamine. Table II and Fig. 3 indicated the incorporation of ^{14}C-N-acetylglucosamine or ^{14}C-glucose into the cell wall β-glucan or chitin-like substance from respective UDP-^{14}C-sugars in the presence or absence mutually different glycan precursors (UDP-N-acetylglucosamine for β-glucan synthesis and UDP-glucose for chitin-like substance synthesis).

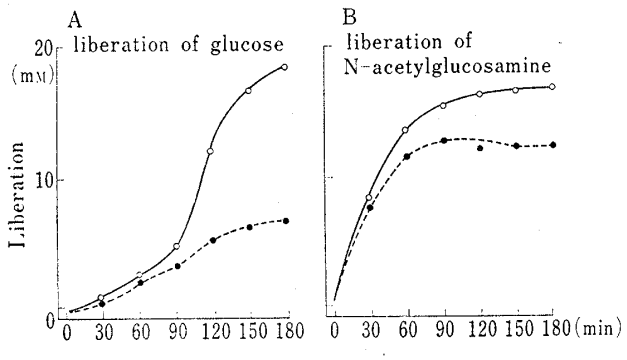

Fig. 1. Effect of UDP-Sugar Derivatives on Liberation of Glucose or N-acetylglucosamine from Cell Wall Glucan or Chitin-like Substance by Crude Enzyme (Under the Conditions of Cell Wall Polysaccharide Syntheses)

detection method: glucose (glucose oxidase), N-acetylglucosamine (Reissig's method) UDP-glu: UDP-glucose, UDP-glcNAC: UDP-N-acetylglucosamine
(A) —○—: enzyme+laminarin
---●---: emzyme+laminarin+UDP-glcNAc
(B) —○—: enzyme+chitodextrin
---●---: enzyme+chitodextrin+UDP-glu

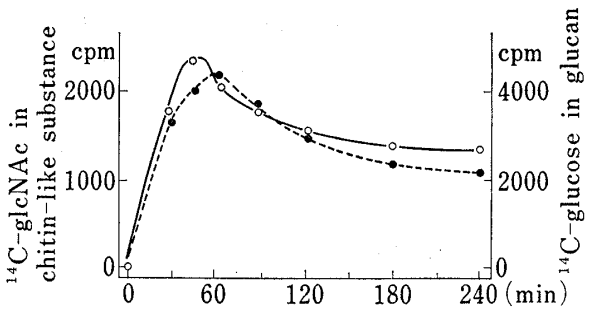

Fig. 2. Incorporations of ^{14}C-glucose or ^{14}C-N-acetylglucosamine from UDP-^{14}C-glucose or UDP-^{14}C-N-acetylglucosamine into Cell Wall Glucan and Chitin-like Substance

(glu: glucose, glcNAc: N-acetylglucosamine)
—○—: ^{14}C-glu, ---●---: ^{14}C-glcNAc

5) H. Nanba and H. Kuroda, *Chem. Pharm. Bull.* (Tokyo), **19**, 448 (1971).
6) H. Nanba and H. Kuroda, *Chem. Pharm. Bull.* (Tokyo), **19**, 1402 (1971).

In the biosyntheses of some bacterial cell wall polymers (peptidoglycan and teichoic acid), Baddiley[7] found that the precursors of peptidoglycan and teichoic acid (UDP-sugars) require competitively the common glycosyl carrier lipid (undeca prenol derivative) to form respective lipid intermediates. As a result of this competition, coexistence of the synthetic systems of peptidoglycan and teichoic acid brought about the considerable curtailment of production of both compounds. While in the case of this fungal cell walls, coexistence of the synthetic systems of β-glucan and chitin-like substance brought about the increase of production of both compounds as shown in Table II and Fig. 3. It is unlikely that the synthetic systems of β-glutan and chitin-like substance require competitively the commom lipid phosphate acceptor like some bacteria.

In the previous paper,[3] the authors proved the presence of hydrolytic enzymes in mycelial crude enzyme. Therefore, the results shown in Table II and Fig. 3 suggest that coexistence of both precursors causes an acceleration of glycan synthetase or an obstruction of hydrolytic enzyme. The localization of synthetases and hydrolytic enzymes on cell wall glycan in mycelial cell was studied by the centrifugation method and the most of enzyme activities were found in the microsomal fraction (β-glucan synthetase and chitin synthetase) and the soluble fraction (β-glucanase and β-N-acetylglucosaminidase) (as shown in Table III).

Table II. Effects of UDP-Sugar Derivatives on Glucan or Chitin-like Substance Synthesis

Additive compound	Incorporation of ^{14}C-labeled compound (cpm)
for glucan	
UDP-^{14}C-glucose+buffer	2273
UDP-^{14}C-glucose+UDP-N-acetylglucosamine	2540
for chitin-like substance	
UDP-^{14}C-N-acetylglucosamine+buffer	639
UDP-^{14}C-N-acetylglucosamine+UDP-glucose	714

Incubation time was 90 min.
Crude enzyme was used as enzyme solution.

Table III. Locarization of Synthetic and Hydrolytic Enzymes of Glucan or Chitin-like Substance in Mycelial Cell

Enzyme fraction	Synthetic enzyme synthesized (cpm)		Hydrolytic enzyme released (mg/ml)	
	Glucan	CLS[a]	Glucose	N-Acetylglucosamine
Nuclear	0	0	0.0	0.0
Mitochondria	0	0	0.0	0.0
Microsome	486	571	0.0	0.0
Soluble	0	0	0.09	0.18
Crude	410	553	0.11	0.15

Incubation time was 90 min.
a) CLS: chitin-like substance

As shown in Table IV, when the microsomal fraction was used as β-glucan synthetase or chitin synthetase, the yeilds of synthesized β-glucan or chitin were almost the same to each other regardless of the presence or absence of UDP-N-acetylglucosamine (in the case of β-glucan synthesis) or UDP-glucose (in the case of chitin synthesis). From the results of Table

7) R.G. Anderson, H. Hussey, and J. Baddiley, *Biochem. J.*, **127**, 11 (1972).

IV, the authors came to the conclusion that the addition of UDP-N-acetylglucosamine to β-glucan synthetic system or the addition of UDP-glucose to chitin-like substance synthetic system gave no effect to these synthetase activities.

The influence of UDP-sugars on β-glucanase or β-N-acetylglucosaminidase in mycelial soluble fraction was shown in Fig. 1. When the laminarin or chitodextrin was incubated with soluble enzyme in the presence or absence of UDP-N-acetylglucosamine or UDP-glucose, considerable decrease of released glucose was observed only in the presence of UDP-N- acetylglucosamine and in the presence of UDP-glucose, amount of the released N-acetylglucosamine was decreased. These results indicate that β-glucanase is inhibited by addition of UDP-N-acetylglucosamine which is a precursor of chitin-like substance and β-N-acetylglucosaminidase is inhibited by addition of UDP-glucose which is a precursor or β-glucan. When the crude enzymes were used as synthetases, the authors indicated in the previous paper,[3] that synthesized glycans secondarily allowed to react with some hydrolytic enzymes which were contained in the crude enzymes. In consequence, as shown in Fig. 2, a little decrease of incorporated radioactivities was observed just after maximum of incorporation curves of ^{14}C-sugars. Judging from the results of Fig. 1, the decreases of incorporated radioactivities which were observed in Fig. 2 will disapper by addition of UDP-N-acetylglucosamine or UDP-glucose into the β-glucan synthetic system or chitin-like substance synthetic system. This exception was confirmed by the experiments shown in Fig. 3. Fig. 3 shows the incorporation profiles of ^{14}C-sugars into cell wall glycans in the presence of UDP-N-acetylglucosamine or UDP-glucose in addition to the crude enzyme and respective radioactive precursors. When the crude enzyme was incubated with ^{14}C-labeled precursor, the incorporated radioactivities of cell wall glycan initiated to decrease later on about 60 min, and the recovery of radioactivities was recognized by addition of UDP-N-acetylglucosamine or UDP-glucose at the time indicated by an arrow (incubation period: 90 min). When the UDP-sugar was added to reaction mixture from the beginning of incubation, the increases of radioactivities of both cell wall glycans were continued still later on 180 min without any decrease. Therefore, it is evident that UDP-N-acetylglucosamine in the β-glucan synthetic system or UDP-glucose in the chitin-like substance synthetic system is not a stimulator of synthetase but an inhibitor of hydrolytic enzymes. It has been considered that hydrolytic enzymes may be involved into overall process of mycelial growth, and Bartnicki-Garcia[8] has presented a hypothesis related to cell wall growth, which consisted

Table IV. Effects of UDP-Sugar Derivatives on Glucan or Chitin-like Substance Synthesis

Additive compound	Incorporation of ^{14}C-labeled compounds (cpm)
for glucan	
UDP-^{14}C-glucose+buffer	3675
UDP-^{14}C-glucose +UDP-N-acetylglucosamine	3661
for chitin-like substance	
UDP-^{14}C-N-acetylglucosamine +buffer	879
UDP-^{14}C-N-acetylglucosamine +UDP-glucose	885

Incubation time was 120 min.
Microsomal enzyme was used as enzyme solution.

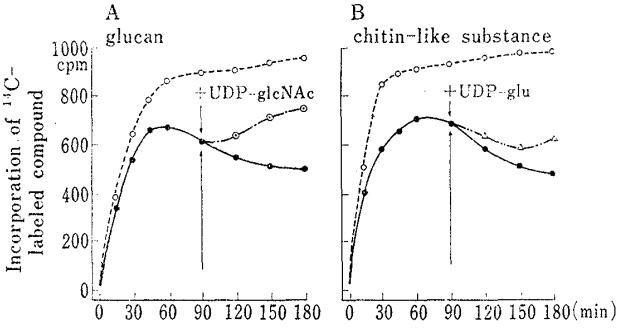

Fig. 3. Effect of UDP-Sugar Derivatives on Glucan or Chitin-like Substance Synthesis

(glucan: incorporation of ^{14}C-glucose, chitin-like substance: incorporation of ^{14}C-acetylglucosamine, glu: glucose, glcNAc: N-acetylglucosamine)
(A) ---○---: UDP-^{14}C-glu+UDP-glcNAc
—●—: UDP-^{14}C-glu
(B) ---○---: UDP-^{14}C-glcNAC+UDP-glu
—●—: UDP-^{14}C-glcNAc

8) S. Bartnicki-Garcia, "Fundamental Aspects of Hyphal Morphogenesis," Vol. 23, Symposium Society of General Microbiology, 1973, p. 245.

of glycan synthetases and hydrolytic enzymes. In this hypothesis, however, he gave no explanation about the possible regulatory mechanism(s) between synthetases and hydrolytic enzymes.

According to the results obtained in this paper, the inhibition of cell wall glycan hydrolytic enzymes by the precursors of the cell wall glycan may be involved into a part of the possible regulatory mechanism(s) described above.

All experimental results demonstrate that the possible regulatory mechanisms of cell wall syntheses is presented as shown in Chart 1.

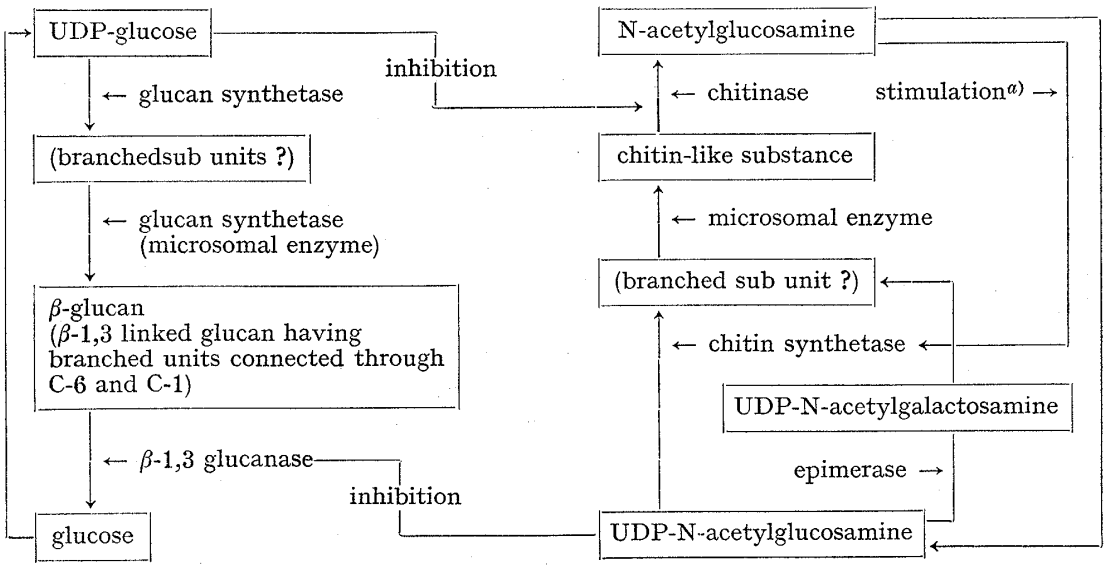

Chart 1. Possible Regulatory Mechanisms of Cell Wall Glycan Syntheses on *Cochliobolus miyabeanus*

a) L. Glaser and D.H. Brown, *J. Biol. Chem.*, **228**, 729 (1957)