STUDIES ON THE MODE OF ACTION OF POLYOXINS. VI*

EFFECT OF POLYOXIN B ON CHITIN SYNTHESIS IN POLYOXIN-SENSITIVE AND RESISTANT STRAINS OF *ALTERNARIA KIKUCHIANA*

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A large number of strains of *Alternaria kikuchiana* that cause the black spot disease on pear were isolated from some orchards of Tottori Prefecture, Japan. From them seven strains, which showed different sensitivities to polyoxin B, were selected and used in the experiments. Polyoxin B, at a concentration of 10 μM, inhibited more than 50% of the incorporation of glucosamine-^{14}C into cell-wall chitin in washed mycelia of the polyoxin-sensitive strains and at the same time resulted in an unusually increased accumulation of UDP-N-acetylglucosamine-^{14}C, which is a precursor of chitin biosynthesis. Both the inhibition of glucosamine-^{14}C incorporation into chitin and the increase of accumulation of UDP-N-acetylglucosamine-^{14}C caused by polyoxin B were moderately lowered with decreasing the sensitivity to the antibiotic of the different strains. Crude preparations of chitin synthetase were obtained from these strains. Polyoxin B strongly inhibited all the enzyme preparations in competition with their substrate UDP-N-acetyl-glucosamine. The *Km* values for the substrate or the *Ki* values for the antibiotic determined in the enzyme reactions differed slightly from each other. These results indicate that polyoxin B acts as an competitive inhibitor of chitin synthetase, which is concerned in the synthesis of cell-wall chitin of *A. kikuchiana*. And they also suggest that the polyoxin-resistance of this fungus is caused by a lowered penetration of the antibiotic through the cell membrane into the enzyme site.

Polyoxins A~M,[1,2] which belong to the class of peptidyl-pyrimidine nucleoside antibiotics, are produced by *Streptomyces cacaoi* var. *asoensis*. This mixture of polyoxins is widely used as an excellent agricultural fungicide. Polyoxin B, especially, is effective against *Alternaria kikuchiana*, that causes the black spot disease on pear.[3] The minimum inhibitory concentration for mycelial growth of this fungus is 12.5 μg/ml (about 25 μM).[4]

In our previous papers,[5~8] we have shown that polyoxins selectively inhibit the synthesis of cell-wall chitin in some filamentous fungi and that the primaty effect of the antibiotics is competitive inhibition of chitin-UDP acetylglucosaminyl-transferase (E. C. 2. 4. 1. 16., chitin synthetase), which catalyzes the transfer of N-acetylglucosamine (GlcNAc) from uridine 5′-diphosphate N-acetylglucosamine (UDP-GlcNAc) to an endogenous acceptor.

Recently, NISHIMURA *et al.*[9] have reported that they discovered a number of polyoxin-resistant strains of *A. kikuchiana* in some orchards of Tottori Prefecture, Japan.

In this paper we have attempted to elucidate the relationship between polyoxin-action

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and polyoxin-resistance by investigating with the polyoxin-sensitive and resistant strains of *A. kikuchiana*, which were isolated from infected tissues of pears in the orchards of the prefecture described above.

Materials and Methods

Polyoxin B was obtained from Kaken Chemical Co., Ltd. Glucosamine-1-^{14}C ($GlcNH_2$-^{14}C) was purchased from Radiochemical Centre, England and UDP-GlcNAc-^{14}C was prepared according to the method described previously.[10] Chitinase and unlabeled UDP-GlcNAc were purchased from Nutritional Biochemicals and Boehringer Manheim GmbH, respectively. *A. kikuchiana* polyoxin-sensitive strains designated as As-4, Ks-8 and Ns-9, and resistant strains designated as Ar-2, Yr-107, Kr-2 and Nr-8 were screened by the spore germination test described below. A sensitive strain Es-1 was obtained from the Horticultural Research Station (Ministry of Agriculture and Forestry) Hiratsuka, Kanagawa, Japan. *Piricularia oryzae* P_2, which was sensitive to polyoxins, was obtained from National Institute of Agricultural Science, Tokyo.

Spore Germination Test: 0.02 ml portions of an aqueou spore suspension (about 1×10^3 spores/ml sterilized water) of the each strain of *A. kikuchiana* were incubated on glass slides at 28°C for 24 hours. Then the number of normally germinated spores was counted under a light microscope.

Mycelial Growth Test: One of the above fungal strains of *A. kikuchiana* was grown on agar plate of the potato-sucrose medium at 27°C for 5 days. Mycelia grown on the plate were cut out by a cork borer of 5-mm diameter and placed on the other agar plates. After incubation of the mycelial matrices at 27°C for 44 hours, the diameters of radial growth were measured.

Preparation of Washed Mycelium: The organisms were grown in Y. G. liquid medium (Difco yeast extract 0.4% and glucose 2%) at 27°C for 40 hours with reciprocal shaking. Mycelia were harvested by filtration and washed with 0.066 M phosphate buffer, pH 6.8 for three times. Experiment on the incorporation of $GlcNH_2$-^{14}C (1 μCi) into UDP-GlcNAc and chitin in the incubation system using the washed myclia was carried out as described previously.[11]

Preparation of Particulate Chitin Synthetase: The washed mycelia of the fungal strains of *A. kikuchiana* (10 g wet wt.) were suspended in 25 ml of 0.05 M tris-malate-NaOH buffer, pH 7.2 contained 10 mM EDTA, 2 mM $MgCl_2$ and 1 mM mercaptoethanol and then disrupted by grinding with quartz sands (powder) in glass homogenizers at 0°C for 15 minutes. These homogenates were centrifuged at $10,000\times g$ for 10 minutes and the supernatant fractions were centrifuged again at $65,000\times g$ for 30 minutes. The precipitates were suspended in 0.05 M tris-malate-NaOH buffer, pH 7.2 contained 0.1 mM EDTA, 2 mM $MgCl_2$ and 1 mM mercaptoethanol and centrifuged at $65,000\times g$ for 30 minutes. The washed particulate fractions were suspended in 1 ml of the above buffer contained 0.1 mM EDTA and 1 mM mercaptoethanol, and used as the enzyme preparations. The protein contents in the preparations from *A. kikuchiana* strains As-4, Ks-8, Ns-9, Es-1, Kr-2, Nr-8, Yr-107 and Ar-2 were 9.0, 17.3, 8.0, 17.7, 22.8, 9.0, 9.4 and 12.0 μg/ml, respectively.

Assay of Chitin Synthetase Activity: Incubation mixtures contained 2.6 mM UDP-GlcNAc-^{14}C (24,000 dpm), 20 mM GlcNAc, 3.2 mM $MgCl_2$, 0.07 mM EDTA, 0.7 mM mercaptoethanol, 60 μl of 0.05 M tris-malate-NaOH buffer (pH 7.2) and 10 μl of the each enzyme preparation in a total volume of 100 μl. The incubation mixtures were kept at 25°C for 10 minutes and the reactions were stopped by heating the mixtures in a boiling water bath for 1 minute. The mixtures were spotted on Avicel thin-layer plates and the chromatography was performed with a solvent system of MeOH-pyridine-acetic acid-water (6 : 6 : 1 : 4). Radioactive products

which remained at origins of the thin-layer plates were cut out and the radioactivities were counted in a Packard Tri-Carb liquid scintillation spectrometer. These products were considered to be chitin, because they were water-insoluble and gave GlcNAc and $GlcNH_2$ by chitinase digestion and 6 N HCl hydrolysis, respectively.

Results

1. Sensitivities of Isolated Strains of *A. kikuchiana* to Polyoxin B

The spore germination test described in "Materials and Methods" was used as a method to evaluate polyoxin-sensitivities of the various strains of *A. kikuchiana*. Previously EGUCHI *et al.*[3] reported that, when *A. kikuchiana* spores are incubated with polyoxin B, the spores germinate at a normal rate, however the growth of their germ-tubes is strongly inhibited. As Table 1 shows that more than 80 % of the growth of the germ-tubes of strains As-4, Ks-8, Ns-9 and Es-1 was inhibited by 2 μM or 200 μM polyoxin B. These germ-tubes formed protoplast-like structures, finally ceased growing and ruptured. While the antibiotic, at the same concentration, inhibited only about 10 % of the growth of the germ-tubes of strains Ar-2 and Yr-107 (Table 1). Thus the first four strains were sensitive and the latter two were resistant to the antibiotic. Strains Kr-2 and Nr-8 indicated intermediate sensitivity (Table 1).

Table 1. Effect of polyoxin B on the spore germination of various strains of *A. kikuchiana*

Strain	Growth inhibition of germ-tube (%)		Sensitivity to polyoxin B
	Addition of polyoxin B		
	2 μM	200 μM	
As-4	98.5	99.3	+++
Ks-8	94.8	93.7	+++
Ns-9	87.6	99.1	+++
Es-1	80.4	87.0	++
Kr-2	55.6	81.3	+
Nr-8	41.5	88.3	+
Yr-107	11.1	12.8	−
Ar-2	5.9	12.8	−

Polyoxin B, at a concentration of 10 μM, inhibited 42 % and at 50 μM inhibited 81 % of the mycelial growth of strain Es-1. The growth inhibition found in strain Yr-107 by the antibiotic was 5 % at 10 μM and 13 % at 50 μM. The protoplast-like structures were also formed by most of the mycelia of strain Es-1 in the presence of the antibiotic.

2. Effects of Polyoxin B on Incorporation of $GlcNH_2$-^{14}C into Chitin

The incorporation of $GlcNH_2$-^{14}C into UDP-GlcNAc and chitin was observed in *P. oryzae* and in the strains of *A. kikuchiana* under the condition given (Table 2). In the sensitive strains over 50 % of the radioactivity usually incorporated into chitin was inhibited in the presence of 10 μM polyoxin B. At the same time there was an increase of accumulation of UDP-GlcNAc, which was obviously caused by the antibiotic. The concentrations of polyoxin B required to inhibit the chitin synthesis in washed mycelia of the sensitive strains of *A. kikuchiana* were similar to those required to inhibit the growth of strain Es-1. The results also showed that, as the sensitivity to the antibiotic of the different strains decreased, both the inhibition of chitin synthesis and the increase of accumulation of UDP-GlcNAc-^{14}C caused by the antibiotic was lowered markedly.

3 Effect of Polyoxin B on Chitin Synthetase

The chitin formation from UDP-GlcNAc-^{14}C by the particulate chitin synthetase preparations

Table 2. Effect of polyoxin B on the incorporation of $GlcNH_2$-^{14}C into both chitin and UDP-GlcNAc in the incubation system using washed mycelia of *P. oryzae* and various strains of *A. kikuchiana*

Strain		Addition of polyoxin B (μM)	Radioactivity incorporated into UDP-GlcNAc (cpm/tube) $\times 10^3$	Radioactivity incorporated into chitin (cpm/tube) $\times 10^3$	Inhibition (%)
A. kikuchiana	Ns-9 +++	0	56.9	658.4	
		10	165.6	207.7	68.5
		200	386.8	54.3	91.8
	Ks-8 +++	0	54.9	273.3	
		10	88.9	79.0	71.1
		200	135.1	23.8	91.8
	As-4 +++	0	63.3	383.2	
		200	136.8	40.8	89.3
	Es-1 ++	0	140.3	342.9	
		10	179.0	157.3	54.1
		200	273.4	43.0	87.5
	Nr-8 +	0	69.1	529.1	
		10	136.3	233.3	55.9
		200	346.6	110.5	79.1
	Kr-2 +	0	5.6	4.4	
		10	6.1	3.9	11.0
		200	6.1	1.7	61.1
	Yr-107 −	0	118.7	746.9	
		10	125.3	651.3	12.8
		200	154.8	567.0	24.9
	Ar-2 −	0	251.7	753.6	
		10	261.7	691.9	8.2
		200	329.2	551.9	26.8
P. oryzae +++		0	99.1	1,209.8	
		10	429.1	545.8	54.9
		200	739.0	103.1	91.5

from the various strains of *A. kikuchiana* was found under the condition given. And more than 90% of the each enzyme activity was inhibited by 50 μM polyoxin B. When the inhibitions of the enzyme preparations by the antibiotic were investigated as a function of UDP-GlcNAc concentration, the results shown in Fig. 1 were obtained. These double reciprocal plots showed that polyoxin B acted as a competitive inhibitor of the enzyme preparations. MICHAELIS constant *Km* for UDP-GlcNAc and inhibitor constant *Ki* for the antibiotic in the each enzyme reaction were calculated from Fig. 1 and shown in Table 3. The *Km* values and the *Ki* values determined were found to be in the range $2.74 \sim 4.26 \times 10^{-3}$ M and $0.89 \sim 1.86 \times 10^{-6}$ M, respectively. As noted previously,[11] the chitin synthetase from *P. oryzae* P_2 is competitively inhibited by polyoxin B and the *Km* and the *Ki* are 3.33×10^{-3} M and 2.95×10^{-6} M, respectively.

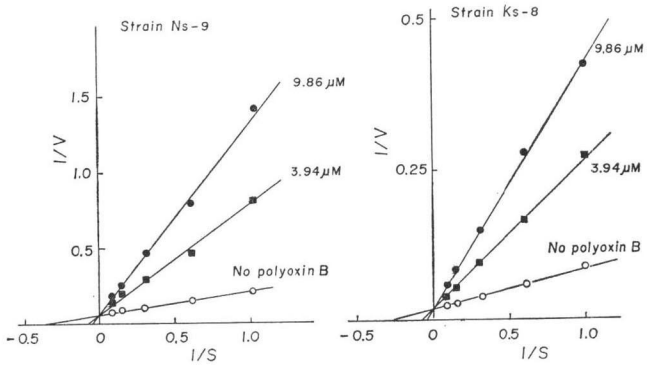
Discussion

The results of the experiments described above support our conclusino[5~8,11] on the mode of

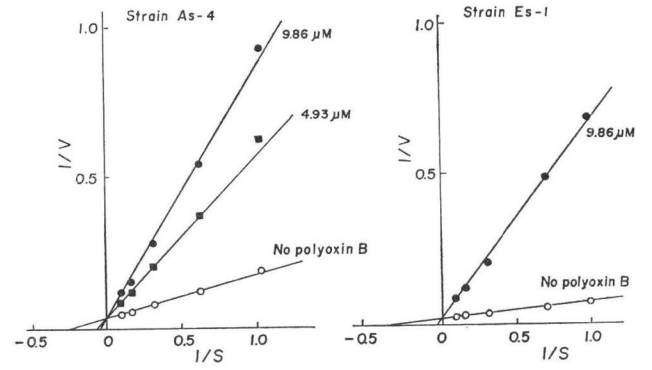
action of polyoxins, namely, they act as competitive inhibitors of chitin synthetase in the synthesis of cell-wall chitin of polyoxin-sensitive fungi. The *Ki* values determined for the inhibition of the chitin synthetase preparations from the different strains of *A. kikuchiana* by

Fig. 1. LINEWEAVER and BURK plots showing competitive inhibition of the chitin synthetase preparations from various strains of *A. kikuchiana* by polyoxin B.

The experiment was carried out as described in the text except that the reaction mixture contained the various concentrations of UDP-GlcNAc-^{14}C. Ordinates show reciprocal reaction velocity (mμmoles/10 minutes) and abscissas show reciprocal substrate concentration (mM). Open circles are reaction in the absence of polyoxin B and solid symbols are with the antibiotic at the concentrations indicated.



(a)



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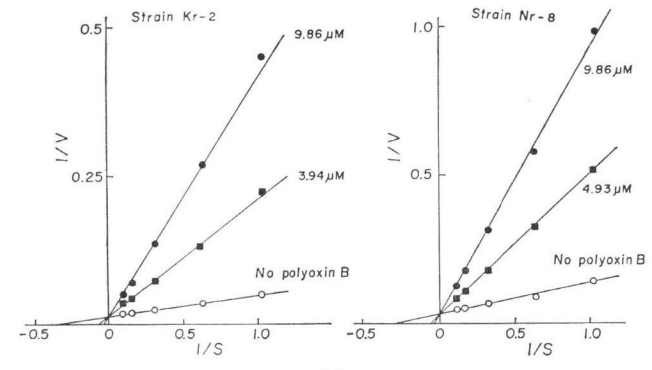

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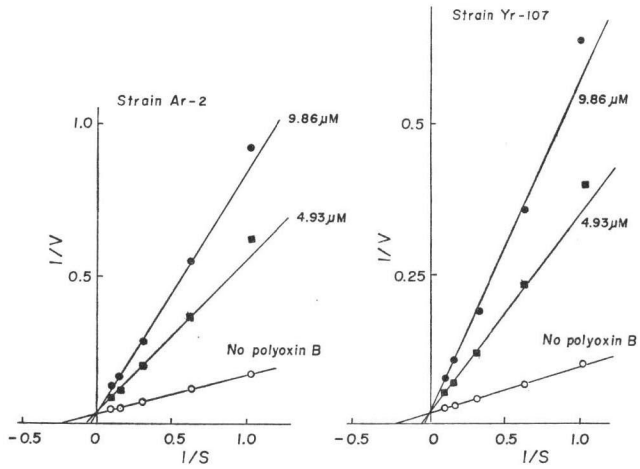

(d)

Table 3. Kinetic constants for chitin synthetase and its inhibition by polyoxin B.

The values of *Km* and *Ki* were obtained from Fig. 1.

Chitin synthetase from *A. kikuchiana*	*Km* for UDP-GlcNAc ($\times 10^{-3}$ M)	*Ki* for polyoxin B ($\times 10^{-6}$ M)
Ns-9	2.74	1.18
Ks-8	4.26	1.77
As-4	4.17	1.59
Es-1	3.33	0.94
Nr-8	3.33	1.24
Kr-2	2.94	0.89
Yr-107	3.85	1.18
Ar-2	4.26	1.86

polyoxin B were comparable to those obtained for the inhibitions of the enzyme preparations from *Neurospora crassa*,[7] *P. oryzae*,[11] *Saccharomyces carlsbergensis*[12] and *Mucor rouxii*.[13] The each *Ki* value was about 1,000 times smaller than the corresponding *Km* value for UDP-GlcNAc.

The degree of the inhibition of $GlcNH_2$-^{14}C incorporation into chitin in *A. kikuchiana* by polyoxin B varied in proportion to the polyoxin-sensitivities of the various strains of this fungus. Thus the chitin synthesis from $GlcNH_2$-^{14}C in the sensitive strains was strongly inhibited, while that in the resistant strains was inhibited to lesser extent by the antibiotic. The results of the kinetic investigation with chitin synthetase, which is the target enzyme of polyoxins, indicate that the polyoxin-resistance is not due to the presence of the enzyme with altered affinity for the inhibitor polyoxin B or the substrate UDP-ClcNAc. The resistance is probably caused by a decrease of polyoxin B concentration on the enzyme site. As noted by KELLER and CABIB,[12] the chitin synthetase of *S. crlsbergensis* is not located on the outside of the cytoplasmic membrane. This suggests that the decrease of the antibiotic concentration described above results from altered permeability of the cells of *A. kikuchiana*. Further studies on the mechanism of polyoxin-resistance are under way by using radioactive polyoxins.

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