Original Article

Mode of Inhibition of Chitin Synthesis by Diflubenzuron in the Cabbage Armyworm, *Mamestra brassicae* L.

Takashi MITSUI, Chieko NOBUSAWA and Jun-ichi FUKAMI

*The Institute of Physical and Chemical Research,
Hirosawa, Wako 351, Japan*

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No morphological difference was found in the epithelial cells of midgut between the normal and the diflubenzuron-treated last instar larvae of the cabbage armyworm, *Mamestra brassicae* L. Diflubenzuron and polyoxin D strongly inhibited chitin synthesis in the peritrophic membranes of the midgut when glucosamine or *N*-acetylglucosamine was applied, as a precursor of chitin, either inside or outside the midgut. When UDP-*N*-acetylglucosamine was injected into the midgut with polyoxin D, chitin synthesis was blocked, but not with diflubenzuron. The results lead to the following speculation: UDP-*N*-acetylglucosamine would be formed from glucosamine or *N*-acetylglucosamine in the epithelial cells and then move to the outer face of the biomembranes of the cells to form chitin. Diflubenzuron may act as an inhibitor of the transport system of UDP-*N*-acetylglucosamine across biomembranes, since an accumulation of UDP-*N*-acetylglucosamine is known in insects treated with diflubenzuron. Na⁺-K⁺ ATPase and Ca²⁺-Mg²⁺ ATPase were not affected by the insecticide.

INTRODUCTION

The insecticide, diflubenzuron (Dimilin, TH60-40), is well known as an inhibitor of insect chitin synthesis but the mode of action of the compound remains unknown. As the final precursor of chitin synthesis, UDP-*N*-acetylglucosamine, was found to accumulate in insects treated with diflubenzuron.^{1–7)} Thus, it has been concluded that the compound blocks the terminal polymerization step in the chitin synthesis pathway.^{4,5,7)} The insecticidal activity of diflubenzuron was thought to be due to direct chitin synthetase inhibition. However, it is of particular interest that the insecticide does not inhibit the enzyme, whereas polyoxin D does.^{7–12)} At present, two hypotheses are proposed for the mode of inhibition of the insecticide.^{8,12,13)} The first one is that the insecticide interferes with the proteolytic activation of the enzyme zymogen¹³⁾ and the second is that it disrupts a critical spatial arrangement of the enzyme complex or interferes with some delicate

regulatory mechanism.^{8,12)} In this paper, we examined the effect of diflubenzuron on chitin synthesis using the midgut epithelial cells as a target and proposed another hypothesis that diflubenzuron affects the transport of UDP-*N*-acetylglucosamine across the biomembranes.

MATERIALS AND METHODS

1. *Experimental Animals*

Cabbage armyworm larvae, *Mamestra brassicae* L., were reared under sterile condition¹⁴⁾ at 25°C under a long day (18L : 6D) photoperiod regimen. Newly molted final instar (6th instar) larvae were used for the morphological observation of midgut and ATPase assay. The head capsule slipped penultimate instar larvae were collected under sterile condition into an individual plastic container with a sterile filter paper on the bottom. The newly molted final instar larvae in the containers were supplied to the midgut culture experiments within 3 hr after molt. As usual the containers were kept at 20°C for about 12 hr

and moved to 25°C to allow them to molt simultaneously.

2. Chemicals

Diffubenzuron, 1-(4-chlorophenyl)-3-(2,6-difluorobenzoyl) urea, was supplied by Nissan Chemical Co., Ltd., Tokyo. The compound in dimethyl sulfoxide was used for all the experiments. Polyoxin D was provided by Kaken Chemical Co., Ltd., Tokyo. D-[1-¹⁴C]-glucosamine hydrochloride (¹⁴C-GA, 60.8 mCi/mmol) and uridine diphospho-*N*-acetyl-D-[U-¹⁴C]-glucosamine, ammonium salt (¹⁴C-UDP-AGA, 275 mCi/mmol) were purchased from The Radiochemical Centre, Amersham, England. Acetyl-D-[1-¹⁴C]-glucosamine (¹⁴C-AGA, 58.18 mCi/mmol) and adenosine 5'-triphosphate, tetra(triethylammonium)salt, [γ -³²P](³²P-ATP) were purchased from New England Nuclear. These compounds were dissolved in distilled water. This stock solution was then added to the medium to give the desired concentration. Other chemicals were all reagent grade.

3. Histological Procedures

Newly molted final instar larvae were fed on the artificial diet containing 10 ppm diflubenzuron (the concentration completely prevented pupation) and the normal diet as control. Midguts were dissected out from the treated and the normal animals at every 24 hr after feeding, fixed in Bouin's, sectioned at 7 μ m and stained with haematoxylin-eosin. Observation was made at 400 $\times$ magnification with a Vanox, Olympus phase contrast microscope.

4. Midgut Culture Procedures

For culture *in vitro*, the procedures were the same as previously reported.^{15,16)} The final instar larvae from the individual containers as described above were surface-sterilized and the midguts were taken out. The precursors of chitin, GA, AGA and UDP-AGA, and the test compounds including the inhibitors of chitin synthesis and ATPase were applied to the midguts following the 3 application methods as shown in Fig. 1.

4.1 Outside application

After posterior end of the midgut was ligated by cotton thread, Grace's medium was injected into the midgut and then, the other

Fig. 1 Application methods of the inhibitors and the precursors to the ligated midguts.

(a): inside, (b): outside, (c): inside-outside.

○: ¹⁴C-precursor, ●: inhibitor, medium: Grace's medium.

end was ligated as well. The ligated midguts were cultured in the Grace's medium in the presence of the inhibitors and the precursors for 16 to 24 hr.

4.2 Inside application

A given amount of the individual inhibitors and precursors was injected into the midgut ligated at the posterior end and the other end was also ligated after injection. The ligated midgut was cultured in the plain Grace's medium.

4.3 Inside-outside application

After the inhibitors were injected into the ligated midgut, it was cultured in the Grace's medium in the presence of the precursor. All procedures were conducted under sterile condition.

5. Analysis of Chitin Synthesis

After *in vitro* culture for 16–24 hr, 6 midguts were homogenized in 10 ml 5% trichloroacetic acid with a glass homogenizer and then centrifuged. The pellets were digested in 2 ml 30% KOH at 100°C for 2 hr and then diluted with 10 ml distilled water. The indigested material was collected on a glass-fiber filter (GC 50) and washed twice with 10 ml distilled water, twice with 10 ml acetone and twice again with 10 ml distilled water. The radioactivity incorporated into chitin was determined by combustion of the indigested material along with the filter using Sample Oxidizer (Packard Tri-Carb B306) followed by scintillation counting (Packard Tri-Carb 460 CD). When the ligated midgut in which the inhibitor was injected was cultured in the presence of the precursors (the inside-outside application), both ends were cut off from the ligated midgut before analysis.

6. ATPase Activity

The method of Anstee and Bell¹⁷⁾ was modified for the assay of Na^+ - K^+ ATPase activity. Midgut, about 2 g, was dissected out from 50 newly molted final instar larvae and homogenized in 20 ml of ice-cold buffer A containing of 250 mM mannitol, 5 mM EDTA, 0.1% sodium deoxycholate in 30 mM histidine-HCl, pH 7.2. The resulting homogenate was then treated with sodium iodide as described by Nakao *et al.*¹⁸⁾ and centrifuged at 25,000 *g* for 30 min at 0°C. The supernatant was centrifuged at 105,000 *g* for 60 min. The resulting pellets were resuspended in 5 ml buffer B (4 mM MgCl_2 , 100 mM NaCl, 20 mM KCl, 0.1 mM EDTA in 10 mM Tris-HCl, pH 7.5) after washing with 10 mM Tris-HCl buffer (pH 7.5) containing of 1 mM EDTA and used as the enzyme. The inhibitor in 50 μl solvent was added to the assay mixture containing a 100 μl aliquot of enzyme source (60–100 μg protein) and 0.85 ml of the buffer B. The reaction was initiated by the addition of 100 μl ^{32}P -ATP (final concentration 0.25 mM). After incubation at 30°C for 20 min, the liberated ^{32}P was measured following the method of Yamaguchi *et al.*¹⁹⁾ For the Na^+ - K^+ ATPase activity of midguts excised from the insects treated with diflubenzuron, newly molted final instar larvae were fed on the artificial diet containing 10 ppm

diflubenzuron and the normal diet as a control, and sacrificed 24 hr after feeding was started.

Ca^{2+} ATPase activity was measured following the method of Yamaguchi *et al.*¹⁹⁾ Preparation of enzyme source was the same as the above except for the sodium iodide treatment. The resulting pellets were suspended in 5 ml buffer C which consisted of 5 mM MgCl_2 , 100 mM KCl, 1 mM EGTA in 10 mM Tris-HCl (pH 7.5). A 100 μl aliquot of enzyme source (about 60 μg protein) was incubated with the inhibitor in 0.85 ml buffer C. The reaction was started by addition of ^{32}P -ATP (final concentration 0.5 mM) and the incubation was carried out at 30°C for 10 min for Mg^{2+} ATPase activity. For Ca^{2+} - Mg^{2+} ATPase activity, 0.47 mM CaCl_2 was added to the reaction mixture. The liberated ^{32}P was measured as above.

RESULTS

1. Histological Observation of Midgut

The midgut was made up of two types of epithelial cells, *i.e.*, columnar and goblet cells, a basement membrane and muscle in newly molted larvae as shown in Fig. 2A. The epithelial cells bear short microvilli inside the midgut and are covered by a thin sheath, that is, peritrophic membrane, which has the same components as the inner layers of the cuticle;

Fig. 2 The section of the midgut of *Mamestra* last instar larvae.

CC: columnar cells, GC: goblet cells, RC: regenerative cells, BM: basement membrane, A: newly molted last instar larvae, B: wandering larvae (5 days after molt).

Table 1 Effect of inhibitors on chitin synthesis in *Mamestra* midguts *in vitro* by the outside application.

Precursor (0.1 μ Ci/ml)	Inhibitor	Concentration (μ g/ml)	14 C incorporated (dpm)	Rate
14 C-AGA	Control		14,681	100
	Diflubenzuron	1	1,979	13.9
	Polyoxin D	5	2,578	17.6
		1	10,833	73.8
	Ouabain	50	14,827	101.0
		5	15,268	104.0
	Heptachlor epoxide	50	14,946	101.8
	Ethacrynic acid	50	16,090	109.6
	Captan	50	13,120	89.4
14 C-UDP-AGA	Control		705	100
	Diflubenzuron	2.5	206	29.2

Table 2 Effect of inhibitors on chitin synthesis in the peritrophic membranes by the inside-outside application.

Inhibitor	Concentration (μ g/ml)	14 C incorporated (dpm)	Rate
Control		3,264	100
Diflubenzuron	1	596	18.3
Polyoxin D	5	875	26.8
Furosemide	50	3,091	94.7
Ouabain	50	2,827	86.6

The ligated midguts, in which the inhibitors were injected, were cultured in the Grace's medium in the presence of 0.1 μ Ci/ml 14 C-AGA.

a basis of chitin with protein incorporated in it.²⁰⁾ At the wandering stage, 5 days after final molt, the small basal cells, regenerative cells, appeared on the basement membrane (Fig. 2B). At pupation, the epithelial cells degenerated and the regenerative cells grew to replace them. So, the larval midgut was completely renewed to the pupal midgut. When the larvae were fed on the diet containing 10 ppm diflubenzuron, pupation was completely prevented but no morphological difference was observed in the epithelial cells between the treated and the normal insects.

2. Inhibition of Chitin Synthesis

When the midguts were treated with the inhibitors by the outside application using 14 C-AGA as a precursor, diflubenzuron and polyoxin D strongly inhibited chitin synthesis in peritrophic membranes as shown in Table 1. Captan showed only 10% inhibition at high

concentration of 50 μ g/ml. Other compounds, ouabain, a typical inhibitor of Na^+ - K^+ ATPase, heptachlor epoxide, an inhibitor of Ca^{2+} - Mg^{2+} ATPase, and ethacrynic acid, an inhibitor of sodium transport, had no effect on chitin synthesis *in vitro*. When 14 C-UDP-AGA was used as a precursor in the outside application, incorporated radio activity was only 705 dpm even in the control. It may be due to poor penetration of UDP-AGA into the cells from outside (blood circulation).

Diflubenzuron and polyoxin D also inhibited chitin synthesis when the inhibitor and precursor were applied following the inside-outside application method (Table 2). Furosemide, another inhibitor of sodium transport, had little effect on it. Ouabain had slight inhibition of it when injected into the ligated midguts, whereas no inhibition was observed by the outside application method as above. In this method, the both ligated ends were cut off

Table 3 Effect of diflubenzuron and polyoxin D on chitin synthesis in the peritrophic membranes *in vitro* by the inside application.

Precursor	Inhibitor	Concentration ($\mu\text{g/ml}$)	^{14}C incorporated (dpm)	Rate
^{14}C -UDP-AGA 0.02 $\mu\text{Ci/animal}$	Control		21,942	100
	Diflubenzuron	0.25	23,785	108.4
		1.0	19,726	89.9
		2.5	21,986	100.2
	Polyoxin D	1.0	7,306	33.3
		4.0	7,329	33.4
		5.0	5,771	26.3
^{14}C -AGA 0.04 $\mu\text{Ci/animal}$	Control		15,986	100
	Diflubenzuron	1.0	3,534	22.1
^{14}C -GA 0.1 $\mu\text{Ci/animal}$	Control		1,978	100
	Diflubenzuron	0.25	438	22.1
		1.0	646	32.7
		2.5	312	15.8

after incubation and the remaining midguts (about 1/3 of total midguts) were supplied for chitin analysis. So, the incorporated radio activity in the control was about 1/3 as much as in the control of the outside application (Table 1).

When diflubenzuron and polyoxin D were injected into the ligated midguts with ^{14}C -UDP-AGA as a precursor, diflubenzuron did not inhibit chitin synthesis at all at the concentration of 0.25–2.5 $\mu\text{g/ml}$, whereas polyoxin D did it (Table 3). On the other hand, when ^{14}C -GA or ^{14}C -AGA was used as a precursor in the inside application, diflubenzuron showed a strong inhibition of chitin synthesis as done by other application methods.

3. Effect of Diflubenzuron on ATPase Activity

From above experiment, diflubenzuron was considered to bring about the change of midgut permeability to UDP-AGA across the bi-membranes of the epithelial cells. Then, the ATPase, an active ion pump, was extracted from the midgut and the enzyme activity was assayed as described in the section for Methods. As shown in Table 4, $\text{Na}^+\text{-K}^+$ ATPase from the midgut was not so sensitive to ouabain, that is, about 30% inhibition at 8.5×10^{-5} M. Ethacrinic acid exerted a slight inhibition but the other compounds, diflubenzuron and oligomycin, gave no inhibition. The midguts were taken out from the larvae fed on the diet containing 10 ppm diflubenzuron for 24 hr after molting. Then the enzyme activity was as-

Table 4 Effect of inhibitors on $\text{Na}^+\text{-K}^+$ ATPase activity.

Inhibitor	Concentration (M)	Activity (nmol/mg protein)	Rate
<i>In vitro</i>			
Control		140	100
Diflubenzuron	3.2×10^{-4}	158	112.9
Ouabain	8.5×10^{-5}	103	73.6
Ethacrinic acid	3.3×10^{-4}	130	92.9
Oligomycin	6.2×10^{-5}	154	110
<i>In vivo</i>			
Control		136	100
Diflubenzuron		131	96

Table 5 Effects of diflubenzuron and heptachlor epoxide on Mg^{2+} ATPase and Ca^{2+} - Mg^{2+} ATPase activity.

Inhibitor	Concentration (M)	Mg^{2+} ATPase		Ca^{2+} - Mg^{2+} ATPase	
		nmol/mg protein/min	Inhibition (%)	nmol/mg protein/min	Inhibition (%)
Control	—	6.9	—	32.2	—
Diflubenzuron	8.0×10^{-6}	6.2	10.1	34.9	0
	8.0×10^{-5}	2.8	59.0	37.1	0
	3.2×10^{-4}	4.3	37.9	35.4	0
Heptachlor epoxide	2.5×10^{-4}	6.8	1.5	25.6	20.5

sayed. There was no difference in activity between the treated and the normal insects.

Diflubenzuron inhibited, as shown in Table 5, Mg^{2+} ATPase at high concentrations, but the effect of inhibition was completely recovered by an addition of Ca ion to the reaction medium. As a result, diflubenzuron had no effect on Ca^{2+} - Mg^{2+} ATPase at all, whereas heptachlor epoxide slightly inhibited the enzyme.

DISCUSSION

The midgut culture system is ideal for the study on the mechanism of chitin synthesis. The epithelial cells are exposed to the midgut cavity as microvilli whereas the epidermal cells adhere to the overlying cuticle. Therefore, in the epithelial cells the inhibitors and the precursors can be directly applied to the outer face of the biomembranes. Diflubenzuron and polyoxin D strongly inhibited chitin synthesis in the peritrophic membranes when applied from either inside or outside of the midguts using GA or AGA as a precursor. On the other

hand, when UDP-AGA was directly applied to the surface of the microvilli, diflubenzuron did not block chitin synthesis, whereas polyoxin D did. These results may indicate that diflubenzuron blocks the formation of UDP-AGA from AGA which is mediated by UDP-AGA pyrophosphorylase. However, this is contradictory to the findings on the accumulation of UDP-AGA in the insects treated with diflubenzuron.

When GA or AGA is applied to the midgut, it should be incorporated into the cells to form UDP-AGA and then, the resulting UDP-AGA should be pumped out to the outer face of the biomembranes of the microvilli (Fig. 3A, B). On the other hand, UDP-AGA applied to the surface of the microvilli need not permeate into the cells but it can be directly used *in situ* for chitin synthesis by chitin synthetase which will be on the outer face of the biomembranes of the microvilli (Fig. 3C). However, it is unknown, so far, where in the membranes the chitin synthetase is located, although it is

Fig. 3 Summary of the roles of diflubenzuron in the epithelial cells.

●: AGA, □: UDP, CS: chitin synthetase, I: inside the midgut, O: outside the midgut, EC: epithelial cell, M: biomembrane.

certain that the enzyme is located on the biomembranes.

Concerning the mode of action of diflubenzuron, several hypotheses are proposed. Horst²¹⁾ reported that diflubenzuron inhibited chitin synthetase *in vitro* from brine shrimp larvae. Insect integumental chitin synthetase, however, is reported to be insensitive to diflubenzuron in *Trichoplusia ni* and *Hyalophora cecropia*¹²⁾ and the result agrees well with the earlier findings on insect gut or whole body enzyme preparations in *Tribolium castaneum*,⁸⁾ *Stomoxys calcitrans*^{9,10)} and *Mamestra brassicae*.^{7,11)} It has also been proposed that diflubenzuron prevents chitin synthesis by interfering with the proteolytic activation of the chitin synthetase zymogen.¹³⁾ Another possible speculation is proposed by Cohen and Casida⁸⁾ that diflubenzuron acts either as chitin synthetase inhibitor via active metabolites formed in the integument or as blocking agent by direct binding to non-chitin synthetase sites important in chitin polymerization and fibrillogenesis.

The results of the present work seem to suggest another mechanism that diflubenzuron may disrupt transport systems of the final precursor, UDP-AGA which is enzymatically synthesized from GA or AGA in the epithelial cells, through biomembranes, although it is obscure whether or not chitin synthesis process in the peritrophic membranes is the same as the integuments. Then, the effect of the insecticide on ATPase activity was checked. However, the enzymes were not affected by diflubenzuron. Other possible transport system will be further studied in future.

IBP and edifenphos, organophosphorus fungicides, were found to inhibit biosynthesis of phosphatidylcholine, a main component of biomembranes.^{22,23)} These results propose another possibility that diflubenzuron might affect the membrane formation and, as a result may disrupt the function of the membranes. Diflubenzuron stimulated chitin synthesis in a fungus, *Pyricularia oryzae*, using protoplasts of the fungus.²⁴⁾ Regarding chitin synthesis, stimulation in fungus but inhibition of it in insect still remains unsolved causing much perplexity, even if diflubenzuron may affect biomembranes.

Captan is reported to inhibit chitin synthesis in peritrophic membranes in *Calliphora erythrocephala*²⁵⁾ and to suppress insect cuticle formation.²⁶⁾ The compound also inhibits insect chitin synthetase in *T. castaneum*,⁸⁾ *T. ni* and *H. cecropia*.¹²⁾ In this experiment, however, it was shown that chitin synthesis was inhibited only to a lesser degree. Ouabain was a weak inhibitor when applied inside but not when applied outside. Probably the midgut is not permeable to the compound through blood circulation.

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要 約

ヨトウガにおける diflubenzuron のキチン合成 阻害機構

満井 喬, 信沢智恵子, 深見順一

ヨトウガ (*Mamestra brassicae*) 終齢幼虫を供試して、中腸の上皮細胞に及ぼす diflubenzuron の影響を組織学的に観察した結果、細胞にはまったく影響がみられなかった。¹⁴C 標識したグルコサミンあるいはアセチルグルコサミンを前駆物質として、*in vitro* で中腸に投与した場合、中腸内に注入しても、培養液中に添加しても、囲食膜のキチン質にとり込まれた。diflubenzuron, polyoxin D はこのとり込みを強く阻害した。¹⁴C 標識した UDP-N-アセチルグルコサミンを前駆物質として、diflubenzuron または polyoxin D とともに中腸内に注入すると、polyoxin D はキチン合成を阻害するが、diflubenzuron では阻害がみられなかった。このことから、diflubenzuron は UDP-N-アセチルグルコサミンの生体膜透過に関与しているものと推察された。しかし、Na⁺-K⁺ ATPase, Ca²⁺-Mg²⁺ ATPase など生体膜のポンプに対してはまったく影響がみられなかった。