Feeding and Molt Inhibition by Azadirachtins A, B, and 7-Acetyl-azadirachtin A in *Rhodnius prolixus* Nymphs

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The antifeedant and antimolting effects of azadirachtins A and B, and of a synthetic derivative, 7-acetyl-azadirachtin A, were examined. Given through a blood meal, the effective dose (ED_{50}) for the antifeedant effect ranged from 25 to 30 µg/ml of blood for the three compounds. ATP, a phagostimulant, also given orally, reversed the antifeedant action of azadirachtin A. The ED_{50} values for molt inhibition were 0.04, 0.015, and 0.45 µg/ml of blood for azadirachtins A, B, and 7-acetyl-azadirachtin A, respectively. The mechanisms of feeding and molt inhibitions by azadirachtin A, B, and its 7-acetyl-azadirachtin A derivative are discussed.

Azadirachtin [1], a tetranortriterpenoid from the neem tree, *Azadirachta indica* A. Juss, causes feeding inhibition and growth disruption in insects of various orders [2–8]. However, azadirachtin (aza) does not induce feeding inhibition in all the holometabolous insects studied so far [6]. It also there interferes with the endocrine system, causing modification and suppression of the ecdysteroid titre [7]. The molt inhibition caused by aza can be reversed by ecdysone therapy [8].

In order to come to a better understanding of azadirachtin interference with the nymphal stage of insects, we have studied its effect on *Rhodnius prolixus*, a haematophagous hemimetabolous insect which has often been used for studying insect physiology. Our findings show that aza acts as an antifeedant and as an antihormonal substance in this insect. Both these effects are distinct and especially that of antihormonal activity is not a result of the reduced blood meal [8].

We here describe the inhibition of feeding and molting by aza A, B, and of 7-acetyl-aza A in more detail and present evidence for their action as inhibitors of blood meal ingestion by *Rhodnius prolixus* nymphs.

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Materials and Methods

7-Acetyl-azadirachtin A

The purification of aza A and B [9, Rembold and Forster, manuscript in preparation] from aza as isolated from neem seed [6] has been described. 109 mg Azadirachtin A and 10 ml acetanhydride were heated under reflux for 30 min. Completion of the reaction was followed by use of HPLC (Shandon RP 18; column 5 mm dia, 20 cm length; isocratic eluent, acetonitrile/water (35:65), 1.5 ml/min). Then the acetanhydride was removed *in vacuo* by use of a rotary evaporator and the dark brown coloured residue was fractionated by TLC (SiO_2-coated glass plates, Merck No. 13894, 0.5 mm, 20 × 20 cm; solvent ethyl ether/acetone (9:1)). 7-Acetyl-azadirachtin A migrated in this system with an $R_f = 0.15$. After elution of this band from the silicic acid with acetonitrile, the derivative was pure. Yield 14.3 mg (13%). λ_{max}, 216 nm (acetonitrile) = 17094. NMR ($CDCl_3$): 1 H (C-1), 4.7, 2 H (C-2) 2.31, 1 H (C-3) 5.5, 1 H (C-5) 3.34, 1 H (C-6) 4.64, 1 H (C-7) 5.2, 1 H (C-9) 3.2, 1 H (C-15) 4.7, 2 H (C-16) 1.3/1.65, 1 H (C-17) 2.3, 3 H (C-18) 2.05, 3 H (C-19) 1.75, 1 H (C-21) 5.6, 1 H (C-22) 5.04, 1 H (C-23) 6.46, 3 H (OMe) 3.7, 3 H (OMe) 3.8, OH (C-14) 3.1, OH (C-20) 2.96, 3 H (Me-4′) 1.78, 3 H (Me-5′) 1.89, 1 H (C-3′) 6.87, 3 H (Ac–C-3) 1.94, 3 H (Ac–C-7) 2.01.

Insects

Fourth-instar nymphs of *Rhodnius prolixus*, reared and maintained as previously described [10, 11], were used throughout this study.

Human blood

Citrated human blood, stored at 4 °C for a maximum of 4 h, was used.

Azadirachtin A, azadirachtin B, and 7-acetyl-azadirachtin A treatments

Following ecdysis all insects were starved for 20–25 days. Aza A, B and 7-acetyl-aza A were dissolved in 1:4 ethanol-saline and added to the blood meal as described previously [8] for oral treatments. In some experiments an artificial diet (20 µl ethanol plus sample, 10 ml of a solution of 30 µg ATP in 0.15 M NaCl) was used. To determine the amount of meal ingested the animals were weighed before and after feeding. All experiments were performed with groups of 30–35 nymphs.

Determination of the effective antifeedant and antimolting doses (ED_{50})

The ED_{50} for feeding and molt inhibition induced by aza A, B and 7-acetyl aza A were determined by computing the linear regression, method of least squares [12] of the ingested meal or percentage of molt inhibition against the dose of the substances.

Results

1. Feeding inhibition by azadirachtin A, B and 7-acetyl-azadirachtin A

The effects of aza A, B, and 7-acetyl-aza A on feeding are illustrated in Fig. 1 and Table I. All these substances induced a strong antifeedant effect. Table I also shows that the effect of aza A on feeding inhibition is independent of the meal quality. Using artificial diet we observed the same ED_{50} as observed for the blood meal. The substances revealed no toxic effect at doses up to 50 µg/ml of blood.

2. Reversal of aza A-induced feeding inhibition by ATP

In order to study the mechanism of antifeedant activity of aza A, we tested its effect in presence of ATP. Table II clearly shows that ingestion of the artificial meals is progressively reduced by addition of aza A in increasing concentrations. Similar data are obtained when the insects were fed on blood meal containing aza A (compare Table II with Fig. 1). If ATP, from 30 µmol to 3000 µmol, is added to the meal, while maintaining the aza A

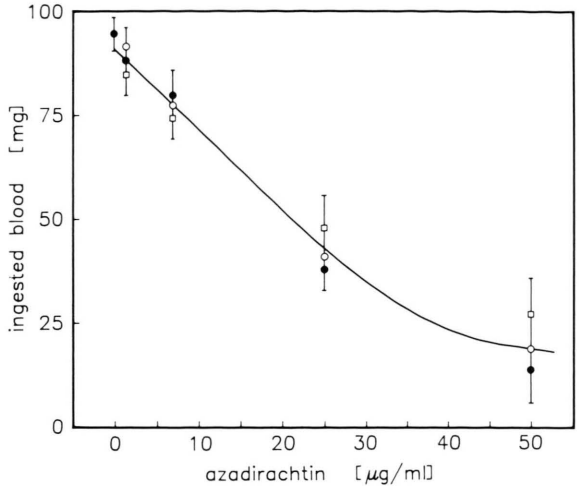

Fig. 1. Effects of azadirachtin A, B, and 7-acetyl-azadirachtin A on feeding if added to the blood meal. (○) Aza A; (●) aza B; (□) 7-acetyl-aza A. Groups of 30–35 nymphs per dose.

Table I. Feeding inhibition of *Rhodnius prolixus* nymphs by azadirachtins A, B, and 7-acetyl-azadirachtin A.

Substances	Effective dose (ED_{50}) [µg/ml]
Azadirachtin A[a]	25.0
Azadirachtin A[b]	27.0
Azadirachtin B[a]	26.0
7-Acetyl-azadirachtin A[a]	30.0

[a] Drugs solved in blood; [b] drugs solved in artificial meal.

Table II. Effect of ATP on feeding inhibition induced by azadirachtin A in *Rhodnius prolixus* nymphs.

ATP [µmol]	Azadirachtin A [µg/ml][a]	Meal ingested [mg]
30	–	98.1 ± 7.1
30	25.0	46.5 ± 6.3
30	50.0	19.9 ± 9.5
300	25.0	85.9 ± 9.1
300	50.0	47.0 ± 7.7
3000	25.0	95.2 ± 5.8
3000	50.0	63.1 ± 7.5

[a] Artificial meal; groups of 30–35 nymphs per dose.

Table III. Molting inhibition of *Rhodnius prolixus* nymphs by azadirachtin A, B, and 7-acetyl-azadirachtin A.

Substances	Effective dose (ED_{50}) [a] [μg/ml]
Azadirachtin A	0.04
Azadirachtin B	0.015
7-Acetyl-azadirachtin A	0.45

[a] The percentage of insects that had molted was determined 30 days after feeding. The ecdysis in the control group reaches 100% on day 16 after feeding.

concentration constant (25 and 50 μg/ml of artificial diet), the amount of meal ingested is increased.

3. Molt inhibition by azadirachtin A, B, and 7-acetyl-azadirachtin A

In the following experiment we compared the effect of aza A, aza B and 7-acetyl-aza A on molt inhibition. Nymphs were treated with blood containing solvent, or with blood containing one of the following concentrations of the compounds: 0.01, 0.05, 0.1, 0.5, and 1.0 μg/ml of blood. The latter doses were not sufficiently high to affect feeding activity. The data collated in Table III show that the ED_{50} for molt inhibition by 7-acetyl-aza A is 0.45 μg/ml, which is a 30-fold higher concentration than for aza B (0.015 μg/ml). In all cases molt inhibition was linearly related to the log of the doses (not shown).

Discussion

These studies show that aza B and 7-acetyl-aza A are as potent as aza A in inducing feeding inhibition when *Rhodnius* nymphs are exposed to feed on blood containing these substances. The ED_{50} for feeding inhibition was practically the same for all the compounds, clearly indicating that minor changes in the chemical structure of aza A do not result in a loss of antifeedant effects.

Chemical inhibition of feeding has been studied in detail for a few phytophagous insects. The mechanisms of action could be (1) a blockade of the input from chemoreceptors normally responding to phagostimulants or (2) by stimulating specific "deterrent" cells or broad spectrum receptors, or through both these mechanisms [14, 15]. Almost without any exception the former mechanism may be reversed by increasing the amount of phagostimulants [16]. Since it has been shown that *R. prolixus* is stimulated by ATP to feed [13], it was interesting to follow the effect of ATP on feeding inhibition as induced by aza A. As shown in Table II, this effect is reversed by increasing amounts of ATP added to the meal. Therefore, it is suggested, that aza A blocks the input of phagostimulant receptors. The latter mechanism has been proposed to explain the antifeedant action of precocene II [17].

Due to its special feeding behavior, *Rhodnius prolixus* can be regarded as a useful model to investigate feeding inhibition. In addition to the antifeedant effect, aza A, B, and 7-acetyl-aza A, given orally, were extremely effective in inducing molt inhibition at much lower concentrations. Table III shows that the ED_{50} for molt inhibition by these substances was 60- to 1800-fold lower than the ED_{50} for feeding inhibition (Table I). These data support the hypothesis that the antifeedant and the antimolting effects of these compounds are distinct from each other as far as their mode of action is concerned. Our results also confirm the observations of Redfern *et al.* [18], Sieber and Rembold [7] and Garcia and Rembold [8]. The data on molt inhibition further indicate that 7-acetylation of aza A was enough of a structural change to cause a great loss of molting inhibition activity. It seems that this acetylation blocked a structural element of aza A which is either important for gut absorption or for the antimolting effect itself.

It should be emphasized that the effects of aza A, aza B and 7-acetyl-aza A on molt are apparently irreversible *i.e.*, no molt was observed even five months after a single treatment, and after at least five full refeedings on blood without containing these substances. It is appropriate to consider that such an effect is due to the interference of aza A, B, and 7-acetyl-aza A with ecdysone production by the prothoracic glands [7, 8].

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