Anti-Juvenile Hormone Activity of Imidazole Compound (KK-22) and Its Diminution by Methoprene in the 4th Instar Silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae)

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1-Citronellyl-5-phenylimidazole (KK-22) was applied topically and orally to and mixed in the diets of 4th instar silkworm larvae, and its anti-juvenile hormone (JH) activity was determined by the induction rates of precocious metamorphosis. Larvae were more sensitive at the earlier stages of 4th instar to KK-22 and there were no significant differences in induction of precocious pupation among the administration methods, topical application, oral ingestion and feeding on diet. Anti-JH activity of KK-22 vanished when methoprene, a JH analogue, was applied immediately after KK-22 treatment.

**INTRODUCTION**

Larval growth, molting, and metamorphosis in insects are generally known to be under the direct regulation of two hormones, juvenile hormone (JH) and molting hormone (ecdysone). Anti-JH activity has been reported on some natural and synthetic compounds, such as kojic acid (Murakoshi, 1972), its related compounds (Murakoshi and Ichimoto, 1972), abietic acid derivatives (Murakoshi et al., 1975), precocenes (Bowers et al., 1976), quinolone alkaloids (Murakoshi et al., 1977), fluoromevalonate (Quistad et al., 1981), ethyl 4-[2-(tert-butylcarbonyloxy) butoxy] benzoate (ETB) and ethyl (E)-3-methyl-2-dodecenoate (EMD) (Staal et al., 1981), and compactin (Monger et al., 1982; Hiruma et al., 1983).

Development of potent anti-JH compounds is expected to provide useful alternative agents for pest control. Recently a new class of compounds with anti-JH activity against silkworms, *Bombyx mori* L. has been found in a group of terpenoid imidazoles (Kuwano et al., 1983, 1984). One of them, 1-citronellyl-5-phenylimidazole (KK-22), was demonstrated to be effective in the induction of precocious metamorphosis in 3rd instar silkworms when administered with food. The effect of KK-22 was dose dependent and was always accompanied by prolongation of the 3rd instar (Asano et al., 1984 a). The action of KK-22 was different from that of precocene I and II in its rate of precocious induction and influence on larval feeding and growth (Asano et al., 1984 b).

In the present study, the induction of precocious pupation by KK-22 in silkworms was investigated in relation to the application stages of the 4th instar, and its reversal by methoprene with JH activity was examined.
MATERIALS AND METHODS

Test insects. A hybrid race of the silkworm (Shunrei x Shogetsu) was reared on an artificial diet, Silkmate-1-S (Nippon Nosan Kogyo Co., Ltd.) in sausage form under 25°C laboratory conditions. The 4th instar larvae eclosed within 24 hr were collected and kept from food until use. Beginning time of feeding was defined as 0 hr in 4th instar.

Test chemicals. An anti-JH active compound, KK-22 (1-citronellyl-5-phenylimidazole) was synthesized according to the procedure reported previously (Kuwano et al., 1984). A JH active compound, methoprene (isopropyl 11-methoxy-3, 7, 11-trimethyl-2, 4-dodecadienoate), was provided by Zocon Co., Ltd.

Bioassays. For dietary administration tests, KK-22 was mixed into an artificial diet in powder form, Silkmate-1-M, as previously reported (Asano et al., 1984 a). KK-22 incorporated into diets was provided 4th instar silkworms at different stages for defined 24 hr periods. Ten larvae were used for each dose.

For topical application tests, both KK-22 and methoprene dissolved in 40 µl of acetone were applied with a microsyringe to the 4th instar larvae at different stages. Ten larvae were used for each treatment.

For oral administration tests, KK-22 dissolved in 3 µl of a mixture (1:1) of methanol and distilled water was orally administered with a microsyringe to 4th instar larvae at different stages. Each treatment used 5–10 larvae.

The induction of precocious metamorphosis and its diminution were evaluated based on the appearance of precocious larvae which spun and made cocoons within the 4th instar. Non-treated larvae of the same generation spun and made cocoons at the end of the 5th instar.

RESULTS

1. Induction of precocious pupation by KK-22

When the diets containing 6.25–100 ppm of KK-22 were given to 4th instar larvae at different stages for 24 hr, precocious pupation occurred depending on dose and stage as shown in Table 1. No larvae died as a result of the treatments with KK-22. Administration of KK-22 within 24 hr after ecysis was most effective in inducing

<table>
<thead>
<tr>
<th>Conc. in diet (ppm)</th>
<th>0–24</th>
<th>24–48</th>
<th>48–72</th>
<th>72–96 (hr)</th>
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<tr>
<td>100</td>
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<td>50</td>
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<tr>
<td>25</td>
<td>80</td>
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<td>12.5</td>
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<td>6.25</td>
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precocious metamorphosis and the induction rate decreased with elapsed stages. No induction was observed at 72–96 hr when committed to the next 5th instar. These results indicated that 4th instar larvae at an early stage were more sensitive to the induction of precocious pupation by KK-22. The sensibility in the second and third 24 hr periods of 4th instar were respectively 1/4 and 1/8 less than that of the first 24 hr, based on the minimum dose level inducing precocious metamorphosis.

Feeding periods of 4th instar larvae in controls were about 4 days, however, the KK-22 treatment prolonged their feeding periods by 2–4 days. Table 2 indicates the

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<tr>
<th>Conc. in diet (ppm)</th>
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<th>24–48</th>
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<td>P</td>
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<tr>
<td>100</td>
<td>7.2*</td>
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<td>8.3*</td>
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<td>50</td>
<td>7.0*</td>
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<td>9.3*</td>
<td>6.7*</td>
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<td>25</td>
<td>7.6*</td>
<td>7.0*</td>
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<td>6.2</td>
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<tr>
<td>12.5</td>
<td>7.0*</td>
<td>5.8</td>
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<td>6.25</td>
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<td>0</td>
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<td>5.4</td>
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* Significant from controls at $p=0.05$.

Precocious silkworms (P): days from the 3rd ecdysis to spinning. Non-precocious silkworms (N): days from the 3rd ecdysis to 4th ecdysis.

Fig. 1. Growth curve of 4th instar silkworms fed on diets containing KK-22 for defined 24 hr (A–D).
Table 3. Precocious pupation induced by topical and oral application of KK-22 to 4th instar silkworms at different stages

<table>
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<tr>
<th>Dose (μg/larva)</th>
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<tr>
<td></td>
<td>0</td>
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<td>by topical application</td>
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<td>2.5</td>
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<td>by oral application</td>
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<td>6</td>
<td>63</td>
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<td>0.6</td>
<td>0</td>
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<td>0.06</td>
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NT: no-test.

developmental periods of larvae destined for precocious pupation (days from the 3rd ecdysis to spinning) and non-precocious pupation (days from the 3rd ecdysis to 4th ecdysis). Non-precocious silkworms observed in treated groups also showed a slight but significant prolongation in their 4th larval instar at high doses of KK-22.

The growth curves of 4th instar larvae are shown in Fig. 1 as average larval body weights of this instar, regardless of whether destined for precocious or non-precocious pupation. Body weight of controls increased in a straight line for the first 72 hr and then reached a plateau because of decreased feeding before the next molting. The growth curve in treatments was similar to that in controls until 72 hr. Thereafter the treated larvae continued to increase their body weight through prolonged feeding periods, so that their weight became markedly larger than that of controls.

To determine the sensitive stage, KK-22 (2.5–40 μg per larva) was topically applied to the 4th instar larvae at different stages (Table 3). Application just after the 3rd ecdysis induced 100% precocious metamorphosis at 40 and 20 μg. However, the induction percentages on 24 hr old larvae decreased to 70% at 40 μg and to 10% at 20 μg. No induction was observed on 48–96 hr old larvae. It is therefore obvious that the 4th instar larvae at early stages are more sensitive to KK-22. All larvae destined for precocious metamorphosis prolonged their feeding period 3–6 days beyond than that of controls and increased their body weight about 40% more.

The results by oral administration of KK-22 (0.06–6 μg per larva) to the 4th instar larvae at different stages are shown in Table 3. Sixty-three and 40% precocious metamorphosis was caused by administration of as little as 6 μg at 0 and 24 hr, respectively. These larvae prolonged the 4th instar period and increased in body weight in a way similar to those described above treated by dietary and topical application.

2. Rescue by methoprene from the action of KK-22

Fourth instar larvae soon after the 3rd ecdysis were topically applied with 20 or 40 μg of KK-22 per larva and then fed on an artificial diet free of KK-22. Methoprene
was topically applied at certain elapsed hours to examine whether it could reverse the precocious pupation by KK-22. The results are presented in Table 4. A single application with 20 and 40 μg of KK-22 induced 67 and 100% precocious metamorphosis, respectively, in this experiment. No induction was caused by a single application of methoprene. However, when methoprene was applied on larvae previously treated with KK-22, the expected precocious pupation declined. The earlier methoprene was applied to larvae treated with KK-22, the more the anti-JH effect of KK-22 was counteracted. No rescue activity was observed with methoprene application 48–96 hr later. However, it is interesting to note that the larvae rescued from the precocious metamorphosis showed similar prolongation to the precocious ones in the 4th instar period for about 2 days.

DISCUSSION

Domestic strains of silkworms are excellent test animals for studying insect endocrinology because of their easy rearing, handling, and genetic homogeneity. In addition, synchronization of larval stages in Bombyx is rather easier than in other insects. Anti-JH activity of KK-22 was found in silkworms (Kuwano et al., 1983). In this study the relationship between application time of KK-22 and precocious induction in 4th instar silkworms was investigated by dietary, topical, and oral application. The results indicated a common trend of greater sensitivity of 4th instar larvae at early stages to the precocious induction of KK-22. JH titer in insects is found to be high at ecdysis to penultimate and final instar (Riddiford, 1980). Therefore, the sensitive stages of 4th (penultimate) instar to KK-22 seem to coincide with the time of high activity of JH synthesis in the corpora allata.

To compare the effective dose levels of KK-22 among dietary, topical, and oral applications, the ingested doses by larvae in dietary application should be estimated. When 4th instar silkworms were reared on artificial diets, the digestibility was reported as 36.7–39.1% (Mukaiyama and Ito, 1962). In this experiment larval body weight in each treatment was recorded daily. Thus, if 40% of ingested diet contributed to weight gain, the amount of diet consumed by 4th instar larvae could be calculated.
Based on the estimated diet consumed, the minimum doses of KK-22 for precocious induction during 0–24, 24–48, and 48–72 hr in dietary application were estimated as 4.2, 15.5, and 62.0 μg per larva, respectively. Comparing these values with those by topical and oral application (Table 3), suggests that the requisite dose level of KK-22 for precocious induction in the 4th instar is similar among the three applications.

Although the mode of action of KK-22 is not yet known, it might involve the inhibition of JH biosynthesis in the corpora allata (Asano et al., 1984 a, b). Murakoshi (1972) reported that the precocious induction of silkworms by kojic acid was observed by dietary administration during 0–72 hr or the entire 4th instar but not during 0–24, 0–48, or later than 72 hr in the instar. Therefore, the anti-JH activity of KK-22 seems different from that of kojic acid. Precocene I and II showed lethal and antifeeding activity to 4th instar silkworms and only a few cases of precocious induction were observed at high doses just below the lethal level (Asano et al., 1984 b). However, KK-22 did not affect larval feeding and growth at doses causing precocious metamorphosis.

An important step of JH biosynthesis in insects is the reduction of 3-hydroxy-3-methyl glutaryl-CoA (HMG-CoA) to mevalonic acid catalyzed by microsomal HMG-CoA reductase (Kramer and Law, 1980). Compactin with anti-JH activity inhibited this enzyme in both rat liver (Endo et al., 1976) and Manduca sexta (Monger et al., 1982). However, KK-22 did not inhibit the microsomal HMG-CoA reductase from rat liver (Mizani, personal communication).

It was previously described that the induction of precocious metamorphosis by KK-22 and its related compounds in the 3rd and 4th instar silkworms was completely blocked by simultaneous application of methoprene (Kuwano et al., 1983, 1984). In this experiment the rescue activity of methoprene was confirmed but it was only effective within 24 hr after application of KK-22 to the newly eclosed 4th instar larvae. This fact suggests that methoprene cannot reverse the precocious induction after 4th instar larva applied with KK-22 have been committed to precocious metamorphosis.

All precocious silkworms induced by KK-22 showed the prolongation of 4th instar and continued feeding during the prolonged larval periods, resulting in body weight greater than that of controls. Similar phenomena were described in the precocious silkworms induced by kojic acid and its related compounds (Murakoshi, 1972; Murakoshi and Ichimoto, 1972). However, despite a prolonged instar and increased body weight in 4th instar by KK-22, some larvae did not enter into precocious metamorphosis and their degree of prolongation and weight increase were less than that of precocious larvae. Therefore, there seem to be critical levels in the prolongation of 4th instar and increase of body weight which are essential to the induction of precocious metamorphosis.

Nijhout (1975) demonstrated that factors associated with somatic size of Manduca sexta larvae play a decisive role in controlling the number of larval instars and hence the onset of metamorphosis. There may exist factors associated with prolongation of 4th instar and abnormal increase of larval body weight which contribute to the induction of precocious metamorphosis by KK-22.

REFERENCES

Asano, S., E. Kuwano and M. Ero (1984 a) Anti-juvenile hormone activity of 1-citronellyl-5-phenyl-


