Effect of an Imidazole Compound, KK-42, on Changes in Trehalose Content and Trehalase Activity in the Silkglands of the Silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

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The fluctuations of trehalose content and trehalase activity accompanying the growth of middle and posterior silkglands of the silkworm, which was controlled by 1-substituted-5-[(*E*)-2,6-dimethyl-1,5-heptadienyl] imidazole (KK-42), was examined. A topical application of KK-42 (50 µg/larva, once a day) to the fifth instar larvae from 3rd through 5th day delayed the spinning for 3 days, although non-treated larvae spun the cocoon silk on day 6. At the spinning, the middle glands of the KK-42-treated larvae were about 1.5 times heavier than those of non-treated larvae. The fluctuation of trehalose content in both middle and posterior silkglands accompanying the growth of silkglands was different between the non-treated and the KK-42-treated larvae. The administration of KK-42 delayed the onset of the activity for 3 days. Trehalase activity in the middle and posterior glands occurred immediately after the spinning, but in the middle silkglands it was about 13 times higher than in the posterior glands of non-treated larvae. Thus, there appears to be a difference in trehalose metabolism between the middle and the posterior silkglands, and, moreover, the prolongation of the silkgland growth by KK-42 may affect their trehalose metabolism.

Key words: trehalose, trehalase, silkgland, imidazole, *B. mori*

INTRODUCTION

The silkglands of the silkworm *Bombyx mori* grow progressively during the middle stage of the fifth instar, and then degenerate immediately after spinning. It has been reported that a topical application of a juvenile hormone analogue (JHA) to the fifth instar larvae results in a prolongation of the fifth instar period and an increase in the weight of the cocoon and its shell (AKAI et al., 1971), due to the stimulation of the growth of the silkglands by the JHA (KAJIURA and YAMASHITA, 1989). Administering a large amount of 20-OH-ecdysone to the fifth instar larvae accelerates the histolysis of the silkglands. Similar phenomena were observed when the anterior part of the silkglands was autolysed in the presence of 20-OH-ecdysone in the culture medium

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(CHINZEI, 1975). These observations indicate that the 20-OH-ecdysone and the juvenile hormone tend to regulate the growth of the silk glands.

Imidazole compound, 1-substituted-5-[(*E*)-2,6-dimethyl-1,5-heptadienyl] imidazole (KK-42) (KUWANO et al., 1985) acts directly on the prothoracic glands to inhibit ecdysone synthesis (YAMASHITA et al., 1987; KADONO-OKUDA et al., 1987). Precocious pupation was induced by the topical application of KK-42 to the fourth instar larvae of the silkworm, *B. mori* (KADONO-OKUDA et al., 1987), but it was not clear how KK-42 affects the growth of silk glands in the fifth instar.

During the growth of the silk glands in fifth instar larvae, the principal sugar, trehalose, accumulates and attains its maximum level just before the spinning of the cocoon. It is consumed rapidly with the histolysis of the glands (SHIMADA et al., 1980; FURUSAWA and YANG, 1991). The decrease of the trehalose content was ascribed to the enhanced activity of trehalase which had localized in silk gland cells (SHIMADA, 1975). In addition, trehalase activity regulates trehalose accumulation in the silk glands by the application of methoprene with juvenile hormone activity (SHIMADA and KAMADA, 1980). Therefore, the fluctuations of trehalose content and trehalase activity may become a good tool for the monitoring the growth and development of the silk glands. Above studies, however, deal with all silk glands, not with the middle and posterior silk glands, irrespective of the different physiological condition in each gland. Investigating the fluctuation of trehalose content and trehalase activity in both glands should contribute greatly to the understanding of silk gland growth.

In this paper, we show that topical application of KK-42 to the fifth instar larvae of the silkworm prolongs the growth of silk glands, and that there seems to be a difference in trehalose metabolism between the middle and posterior silk glands in accordance with the growth of glands through the action of KK-42.

MATERIALS AND METHODS

Insect used. Silkworm larvae, *Bombyx mori* (Kinshu × Showa), were reared on an artificial diet from the first through the third instar at the Institute Experimental Farms. The fourth and fifth instar larvae were reared on mulberry leaves at room temperature (about 27°C).

Topical application of KK-42. 1-benzyl-5-[(*E*)-2,6-dimethyl-1,5-heptadienyl] imidazole (KK-42) (KUWANO et al., 1985) was dissolved in acetone at a concentration of 5 µg/µl. Ten µl of this solution was topically applied along the dorsal vessel of the larva once a day from the 3rd through the 5th day of the fifth instar.

Silk gland sample. Silk glands collected from 30 larvae of the fifth instar and spinning larvae were washed in cold physiological saline (0.75% NaCl) to remove hemolymph. These glands were divided into middle and posterior silk glands, rinsed in the saline, blotted onto glassine weighing paper to remove excess water, and immediately weighed gravimetrically.

Determination of trehalose. Silk glands (30 mg) were homogenized with 100 µg of pentaerythritol as internal standards and a small volume of 80% ethanol in an agator mortar on an ice bath. The homogenate was centrifuged at 3000 rev./min for 15 min. The extraction was repeated 3 times and the combined extracts were evaporated to dryness under reduced pressure in a freeze-dryer. The dry residues were mixed with 100 µl of the mixture of pyridine (5 ml), hexamethyldisilazane (1 ml), and trimethyl-

chlorosilane (0.5 ml), and heated at 75°C for 40 min. The samples were analysed on a column (3 m × 3 mm i.d.) packed with 2% silicon (GE-SE 30 chromosorb W), the temperature increasing from 150°C to 280°C at a rate of 5°C per min. This resulted in the following retention times: 4.8 min for pentaerythritol, 12 min for glucose, and 28.6 min for trehalose. The contents of trehalose was calculated in reference to internal standards.

Assay of trehalase activity. Two g of silk glands was homogenized in an agator mortar on an ice bath with 10 ml of distilled water. The homogenate was centrifuged at 3,000 rev./min for 15 min, and the supernatant was used as crude enzyme solution. The reaction mixture contained 0.3 ml of 50 mM trehalose, 0.3 ml of crude extracts, and 0.3 ml of 0.1 M acetate buffer (pH 5.6). After incubation for 30 min at 37°C, the reaction mixture was immersed in boiling water for 10 min, and then cooled in water. The coagulated protein was centrifuged off as required. To estimate the content of reducing sugars, 200 µl of the solution was mixed with 3 ml of Glucose B-Test WAKO (GOD method) (Wako Pure Chem. Ind. Ltd.) according to the instructions of the supplier. Protein was determined by the method of LOWRY et al. (1951) using bovine serum albumin.

RESULTS

Figure 1 shows the growth of middle and posterior silk glands from non-treated and KK-42-treated larvae, which was expressed in terms of fresh wet weight. In non-treated larvae, the growth profile of the middle silk glands exhibited a sigmoidal curve from day 0 (newly ecdysed larvae) through day 6 (spinning of cocoon). The weight of the glands reached a maximum level of 953 mg/larva at the spinning, and then decreased rapidly to about 220 mg on day 9. In the larvae treated with KK-42, the weight of the middle silk glands was lower than that of non-treated ones from day 4 through day 6, but subsequently it increased to about 1336 mg on day 9 at the spinning of cocoon, and decreased rapidly thereafter, indicating the histolysis of the glands during larval-pupal metamorphosis.

The weight of the posterior silk glands in non-treated larvae began to increase 2 days earlier than in the middle silk gland, attaining a maximum of about 295 mg at the onset of the spinning, and then decreasing rapidly. In contrast, administering KK-42 delayed both the growth of the posterior silk gland and also the spinning for 3 days. The weight of the posterior silk glands was at a maximum level of 251 mg at the spinning. This result demonstrated that KK-42 retarded the growth of the middle and posterior silk glands, which depends on increasing the weights of both glands up to the spinning, comparing with those of non-treated larvae.

To analyze a relation between the fluctuation of trehalose content in the middle and posterior silk glands and their growth, we investigated the changes in trehalose content of both silk glands of non-treated and KK-42-treated larvae (Fig. 2).

The trehalose content in the middle silk glands of non-treated larvae increased up to day 8 (2 days after the day of spinning). The volume of the accumulated trehalose was about 1.7 times higher in KK-42-treated larvae than that of non-treated ones. Thereafter, trehalose decreased rapidly during larval-pupal metamorphosis in both non-treated and KK-42-treated larvae.

In the posterior silk glands of non-treated larvae, trehalose increased up to a max-

Fig. 1. Effect of KK-42 on growth profile of middle and posterior silk glands during the fifth instar. KK-42 ($50 \mu\text{g}/\text{larva}$) was applied topically once a day to the fifth instar larvae from day 3 through day 5. Control (non-treated larvae) began to spin cocoons on day 6 of the fifth instar and pupate on day 10. ■: middle silk glands of control; □: middle silk glands of KK-42-treated larvae; ●: posterior silk glands of control; ○: posterior silk gland of KK-42-treated larvae. Each point represents mean values from 20 to 35 larvae.

imum level of $6.2 \mu\text{mol}/\text{g}$ silk glands on day 4 (2 days before the spinning) and then decreased rapidly to $2.9 \mu\text{mol}$ on day 5. This trehalose decreased gradually until day 9. In the posterior silk glands of KK-42-treated larvae, a similar fluctuation was observed until day 5. However, the trehalose again increased to a level of $5.2 \mu\text{mol}$ on day 10, and decreased rapidly until day 12. Each profile in the posterior silk glands of non-treated and KK-42-treated larvae was different from that in the middle silk glands.

Figure 3 shows the comparison of the change of trehalase activity in the middle and posterior silk glands of non-treated larvae with that of KK-42 treated ones. In the middle silk glands of non-treated larvae, trehalase activity remained at a low level until day 6, and then appeared rapidly from day 7 through day 8 when the weight of the middle silk glands began to decrease (Fig. 1) and trehalose content reached a maximum level (Fig. 2). This activity seemed to decrease from day 8 through day 9 when trehalose began to decrease. In the middle silk glands of KK-42-treated larvae, tre-

Fig. 2. Effect of KK-42 on changes in trehalose content of the middle and posterior silkglands during the fifth instar. Experimental conditions and symbols were the same as in Fig. 1. Each point represents a mean value from three experiments.

halase activity remained almost at a constant level but appeared from day 10, which coincided with the time of the decrease in the weight (Fig. 1) and trehalose content (Fig. 2) of the middle silkglands.

In the posterior silkglands of non-treated larvae, trehalase activity which was at a low level until day 4 appeared slowly until day 6. This appearance of the activity occurred simultaneously with the accumulation and consumption of trehalose from day 2 through day 5 as shown in Fig. 2. From day 6 through day 7, the activity elevated rapidly, and thereafter decreased, accompanied with decreases in weight (Fig. 1) and trehalose content (Fig. 2) in the posterior silkglands (although trehalase activity was lower in the posterior than in the middle silkglands).

In the posterior silkglands of KK-42-treated larvae, the activity maintained a low level until day 9, though it exhibited a rapid increase from day 4 through day 5 when trehalose content decreased rapidly as shown in Fig. 2.

These results demonstrated that an administration of KK-42 to the fifth instar larvae delayed the appearance of trehalase activity along with the growth of silkglands.

Fig. 3. Effects of KK-42 on changes in trehalase activity of the middle and posterior silk glands during the fifth instar. Experimental conditions and symbols are the same as in Fig. 1. Each point represents a mean value from three experiments.

DISCUSSION

We examined the growth of silk glands in the fifth instar larvae which received a topical application of KK-42 (KUWANO et al., 1985), which is an inhibitor of ecdysone synthesis in prothoracic glands of the silkworm (KADONO-OKUDA et al., 1987). An administration of 50 μ g KK-42/larva once a day from day 3 through day 5 delayed the onset of spinning for 3 days, compared with the spinning on day 6 in non-treated larvae (Fig. 1). When the concentration of ecdysteroid in hemolymph was estimated as a peak area on chromatogram using high performance liquid chromatography (data not shown), 20-OH-ecdysone increased rapidly from day 7 in non-treated larvae and day 10 in KK-42-treated ones. This fluctuation is similar to ones observed by CALVEZ et al. (1976) and KIGUCHI et al. (1985) who showed that ecdysteroid level rises around the start of spinning. Thus, the delay of ecdysone secretion from prothoracic glands by KK-42 application may result in the prolongation of the growth of silk glands and 5th instar period.

As for the relation of trehalose to the growth of silk glands, SHIMADA et al. (1980) reported that the trehalose accumulates with the growth of the glands during the fifth instar, and reaches a maximum immediately before spinning the cocoon when the whole silk gland is used. In our experiments, we used middle and posterior silk glands separately as materials (Figs. 1 and 2). Trehalose in the middle silk glands reached a maximum 2 days after the spinning in non-treated larvae and immediately after the spinning in KK-42-treated larvae. In posterior silk glands, trehalose accumulation occurred 2 days before the spinning in both non-treated and KK-42-treated larvae. Thus, a different pattern of trehalose fluctuation was observed between the middle and posterior silk glands, and also between treated and non-treated larvae. Therefore, we can not monitor the growth of the middle and posterior silk glands by means of fluctuation of trehalose content.

Growth prolongation of silk glands by KK-42 resulted in more elevated accumulation of trehalose in the middle gland and further accumulation in the posterior gland around day 9, comparing with those in non-treated larvae (Fig. 2). Trehalase activity was also delayed by the application of KK-42 (Fig. 3), and caused a decrease in trehalose content after the spinning (Fig. 2). This suggests that a topical application of KK-42 prolongs the period of trehalose accumulation, and presumably delays the utilization of trehalose as an energy source for histolysis of the silk glands during larval-pupal metamorphosis.

An interesting phenomenon was observed in the posterior silk gland, where trehalose content rose up through day 4, and then decreased until day 5, even with a trace level of trehalase activity before the larval-pupal metamorphosis. To what does this accumulation and consumption of trehalose contribute physiologically? Although posterior silk glands begin to produce fibroin actively from day 4 (SHIMURA, 1984), it is questionable, because of a trace level of trehalase activity, that the accumulated trehalose contributes as an energy source to synthesize this protein in the posterior silk gland. ASAKURA et al. (1990) reported that the addition of sorbitol-6-phosphate (S-6-P), which is present at higher concentration than trehalose in hemolymph of fifth instar larvae (SAKAMOTO and HORIE, 1979; ASAKURA et al., 1988), to posterior silk gland in culture medium is effective for maintaining the physiological activity of the gland and silk fibroin production. Therefore, S-6-P, rather than trehalose, can be utilized as an energy source for the growth and fibroin synthesis in the posterior silk glands.

SHIMADA (1975) and SHIMADA et al. (1980) have reported that trehalose and trehalase are separately localized in liquid silk and in the silk glands, respectively. We also detected trehalase activity in silk gland which was separated by liquid silk (unpublished data). Our data in this experiment indicate the different fluctuations of trehalose content in liquid silk and trehalase activity in gland cells between the middle and the posterior silk glands, and between non-treated and KK-42 treated larvae. Work is presently proceeding to clarify the physiological functions of trehalase and trehalose in silk glands with relation to the movement of liquid silk from posterior, through middle silk gland, to cocoon filament, which has trehalase activity.

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