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Enzymatic Properties of the Midgut Amylase Activity and its Changes during Development in the Cabbage Armyworm, *Mamestra brassicae* L.

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Abstract Enzymatic properties of amylases in the midgut tissue and lumen contents of the cabbage armyworm, *Mamestra brassicae* L., were determined and the values were compared with those of amylases in the midgut tissue and digestive juice from the larvae of the silkworm, *Bombyx mori* L. Amylases in the midgut tissue and lumen contents showed high activities during the period of feeding in the last instar larvae and no activity during the period of non-feeding after the gut-purge or in the pupal stage. Amylase activity in the midgut lumen contents was markedly greater than that in the midgut tissue. Activities of these digestive amylases showed cyclical rhythms, although the phases were slightly different. Activities were greater in the light period than in the dark period. There is a cyclical rhythm in feeding activity, and the relationship between the actitvities of digestive amylases and feeding activity was discussed.

Introduction

Many researches have been carried out on the digestive enzymes of adults and larvae of various insects (HOUSE, 1974; JANDA & MUNZAROVA, 1980). There is, however, little information on the physiological properties of the midgut amylase in the larvae of the cabbage armyworm, *Mamestra brassicae* L.

In this work, the relationship between the activity of the midgut amylase and feeding activity was investigated and the enzymatic properties of the midgut amylases in the larvae of the cabbage armyworm were determined.

Materials and Methods

1) Experimental animals: *Mamestra brassicae* was reared on an artificial diet (AGUI *et al.*, 1975) at 25°C in a 16L: 8D photoperiod (normal condition) under aseptic conditions until the 4th larval instar, and then semiaseptically until the 6th final larval instar. In some experiments, the 6th instar larvae were reared under continuous light condition. All larvae become non-diapausing pupae. In these experiments, the gut-purge occurred on the 6th day after the last larval ecdysis.

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2) Determination of the diet intake and amount of feces: Fifty 6th instar larvae were reared in small plastic boxes (23×13×6 cm) and cubes of artificial diet were given at the rate of 30 g per box. Feces and residual diets were gathered every 4 hr or every 8 hr and weighed. As a control group, diets were put in the same box for 4 hr or 8 hr without insects and the weight loss determined. This value was utilized for the compensation of naturally reduced weight of residual diets in the experimental group.

3) Isolation of midgut: Larvae were longitudinally dissected in saline every 4 hr from the last larval ecdysis, the midguts were ligated at both ends, separated from foregut and hindgut, blotted on paper filters, and stored at −20°C.

4) Enzyme preparation: One midgut was dissected in 40 ml of 0.01 M NaCl-1/15 M glycine-NaOH buffer (pH 9.5) and the lumen contents were washed out. The buffer solution containing the lumen contents of each midgut was centrifuged for 10 min at 3,000 r.p.m. and the supernatant was used as an enzyme preparation of midgut content of each larvae. For midgut tissue samples the lumen contents were washed out three times with saline solution and excessive moisture was removed on paper filters. Then, the midgut tissue was homogenized with a motorized teflon pestle in 1.0 ml of 0.01 M NaCl-1/15 M glycine-NaOH buffer solution, the homogenate centrifuged at 3,000 r.p.m. for 20 min and the supernatant employed as the enzyme preparation of midgut tissue.

5) Amylase assays: Amylase activity was colorimetrically determined by the same method as previously described (TANABE and KUSANO, 1984). The reaction mixture contained 0.4 ml starch solution (2 mg starch/ml of 1/15 M glycine-NaOH buffer, pH 9.5), 0.1 ml of 0.1 M NaCl and 0.1 ml of enzyme solution. Each enzyme sample was assayed 3 to 4 times and these values were averaged. No amylase activity in the artificial diets was detected.

Amylases from the midgut tissue and lumen contents were determined in the following experimental groups: i) starvation for 32 hr beginning at 8:00 on the 1st day of the last instar under the normal photoperiod, ii) *ad libitum* feeding under continuous light on the 2nd day of the last instar, and iii) *ad libitum* feeding during the light period and starvation during the dark period with the normal photoperiod beginning on the 1st day of the last instar. Also, during the continuous light condition on the 2nd day of the last instar, the amount of food consumed and feces produced were determined.

6) Enzymatic characterization of midgut amylase: Isolation of the midgut tissue and its enzyme preparation were described above.

Enzymatic properties of the midgut tissue amylase were analyzed as follows, i) effect of temperature: 0.4 ml of soluble starch solution (2 mg starch/ml of 1/15 M glycine-NaOH buffer, pH 9.5), 0.1 ml of 0.1 M NaCl and 0.1 ml of enzyme solution; ii) effect of NaCl: 0.4 ml of soluble starch solution (2 mg starch/ml of 1/15 M Tris-HCl buffer, pH 9.5), 0.1 ml of NaCl solution ($10^{-4}\sim 1$ M) and 0.1 ml of enzyme solution; iii) effect of pH: 0.4 ml of soluble starch solution (2 mg starch/ml of

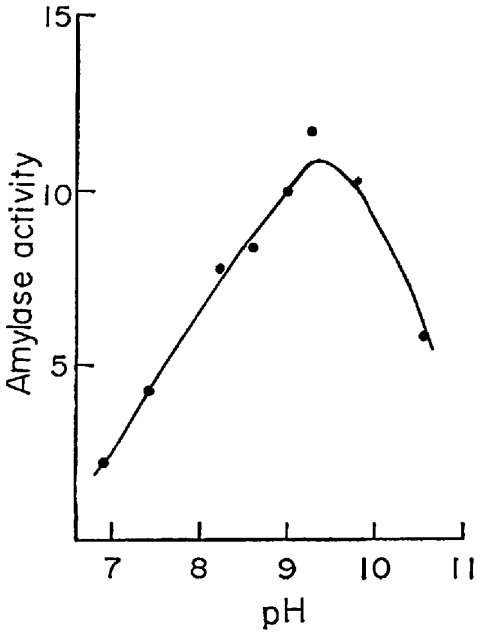

Fig. 1. Effect of pH on the midgut tissue amylase activity of *Mamestra* larvae. Ordinate: enzyme activity (optical density).

distilled water), 0.1 ml of various pH of 1/15 M glycine-NaOH buffer (pH 8.0–10.5) or 1/15 M phosphate buffer (pH 6.9–8.0) and 0.1 ml of enzyme solution.

7) Polyacrylamide gel electrophoresis: Polyacrylamide gel (pH 9.4) of 7.5% was prepared according to a modification of DAVIS's method (NAGAI, 1966 a, b).

Midgut tissue from each individual was homogenized in 0.1 ml of 1/15 M glycine-NaOH buffer solution (pH 9.5), the homogenate was centrifuged for 20 min at 3,000 r.p.m. and the supernatant was used as a midgut tissue enzyme preparation. This solution was mixed with an equivalent volume of 40% sucrose solution. The sample from 50 to 100 μl per one experiment was subjected for electrophoresis. The procedure for the electrophoresis of these enzyme preparations was described previously (TANABE & KUSANO, 1984).

Results

1. *Some enzymatic properties of midgut tissue amylase*

1) Optimal pH range: The midgut tissue amylase was active in the alkaline region and its optimal pH was about 9.5 (Fig. 1).

2) Velocity of hydrolysis: The relationship between concentration of substrate and enzyme activity is shown in Fig. 2. This curve was plotted according to the procedure of LINEWEAVER & BURK (1934). The Km value was 0.27 mg/ml.

3) Optimal temperature: As shown in Fig. 3, the activity of migdut tissue amylase increased with the increase of incubation temperature until 50°C and decreased rapidly at higher temperatures. Accordingly, the optimal temperature for the activity of the midgut tissue amylase was about 50°C.

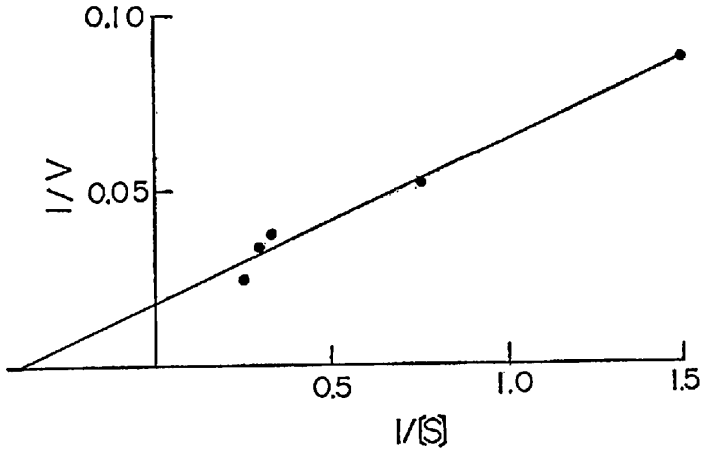

Fig. 2. Relationship between concentration and enzymatic hydrolysis of starch with the midgut tissue amylase of *Mamestra* larvae. Abscissa: 1/s (%). Ordinate: 1/v (μg apparent maltose/min/midgut).

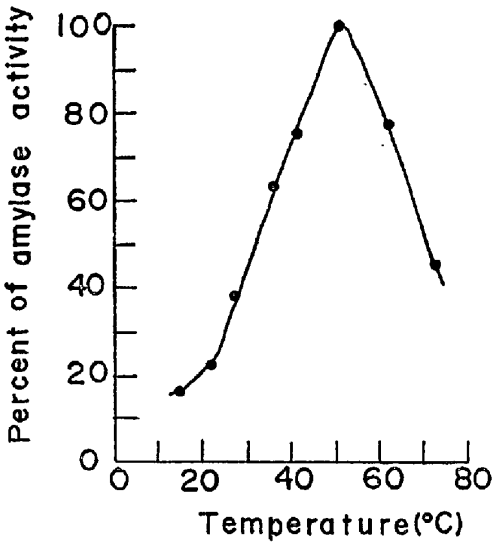

Fig. 3. Effect of temperature on the midgut tissue amylase activity of *Mamestra* larvae. Ordinate: the relative value of amylase activity at various temperature against that of control, 52°C, that was expressed as 100%.

4) Effect of sodium chloride: The midgut tissue amylase was slightly activated by the addition of NaCl in a range from 10^{-3} M to 10^{-5} M and its activity showed the highest increase of 10% at 10^{-4} M.

5) Electrophoresis of midgut tissue and midgut content amylases: As shown in Fig. 4, the digestive amylases on the 3rd day of the last instar larvae were electrophoretically separated into four bands, which were designated A, B, C and D from the cathodal to the anodal end. In the case of the midgut tissue amylase, the activities of the band A were high, and other bands B, C and D were weak activity. In the case of the midgut content amylase, the activity of four bands decreased in the order toward from band A to band D.

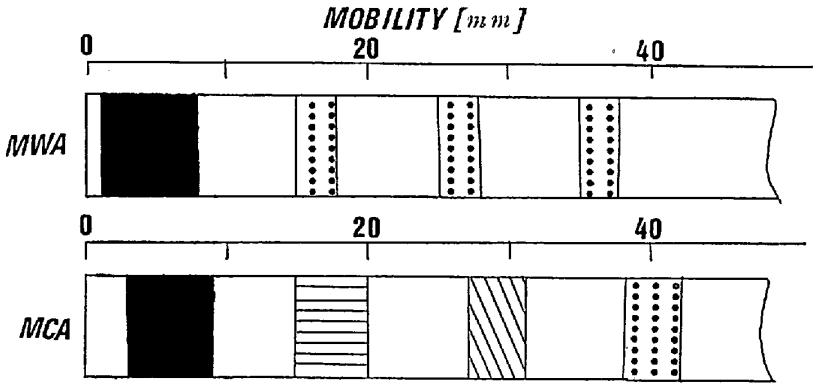

Fig. 4. A diagram showing electrophoretic patterns of the midgut tissue and the midgut content amylases in the larvae of *Mamestra*. ▨: Weak, ▧: moderate, ▤: strong, ■: very strong, MWA: midgut tissue amylase, MCA: midgut content amylase. The test was carried out on the 3rd day of the last instar.

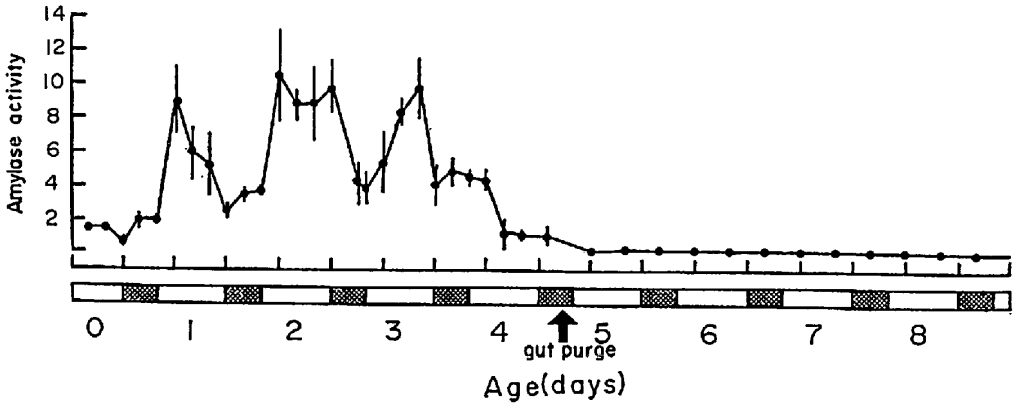

Fig. 5. Changes in amylase activity of midgut tissue in the last instar larvae of *Mamestra*. ⧫: Standard deviation of mean value of seven individuals as one group. Amylase activity: μg apparent maltose/min/midgut; ▨: dark period, □: light period.

2. *Changes in midgut amylase activity*

The midgut tissue amylase activity increased markedly from the 1st day to the 2nd day in the 6th instar, reached the highest level at the light period of the 2nd day, and then, decreased markedly toward the gut-purging stage (Fig. 5). From the gut-purging stage to the pupal stage no amylase activity was detected. A rhythmical fluctuation of the amylase activity occurred and it was three to five times more active during the light period than the dark period. The result shows that there is a cyclical change in the midgut tissue amylase activity in the last instar.

A fluctuation of amylase activity in the midgut lumen contents is shown in Fig. 6. In the period from the 3rd day to the 4th day in the last instar, amylase activity was markedly high. In a range from the 1st day to the 5th day in the last instar larvae the activity showed a rhythmical fluctuation like that found in the

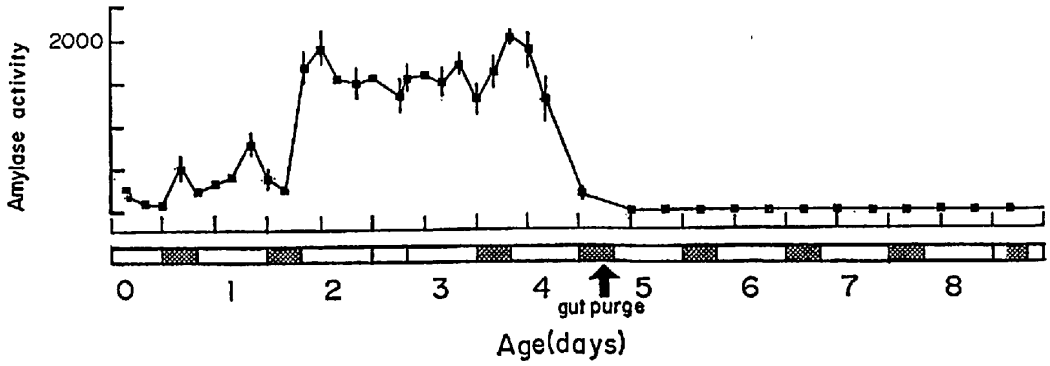

Fig. 6. Changes in amylase activity of midgut lumen contents in the last instar larvae of *Mamestra*. ⊕: Standard deviation of mean value of seven individuals as one group. Amylase activity: μg apparent maltose/min/midgut; ▨: dark period, □: light period.

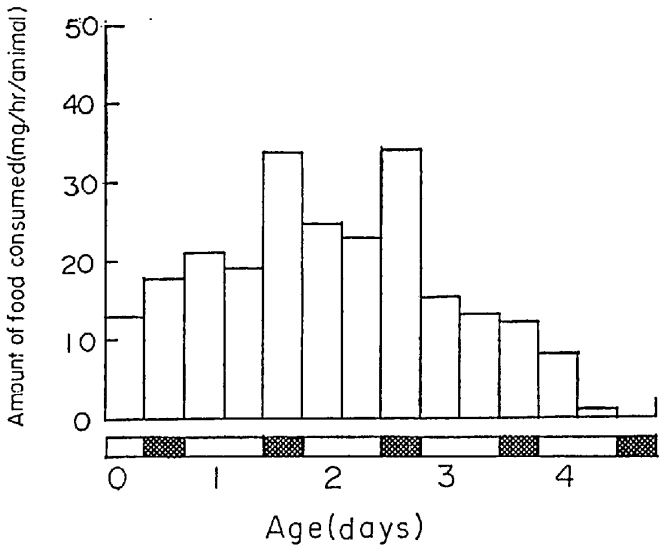

Fig. 7. Rhythm of feeding activity in the last instar larvae of *Mamestra*. ▨: Dark period, □: light period.

midgut tissue. However, the difference in activity between the dark and the light periods was not remarkable. Activity in lumen contents is always much higher than that in the midgut tissue according to Figs. 5 and 6. At the period from the 5th day of the last instar larvae to the gut-purging stage, the amylase activity of the midgut contents decreased sharply and afterwards the amylase activity was hardly detected. It is clear that a phase of the cyclical change of the activity in the midgut content amylase is slightly later than that in the midgut tissue amylase based on the comparison of Figs. 5 and 6.

3. *Cyclical change of feeding activity*

A fluctuation of the amount of food intake from the 1st to the 4th day in the

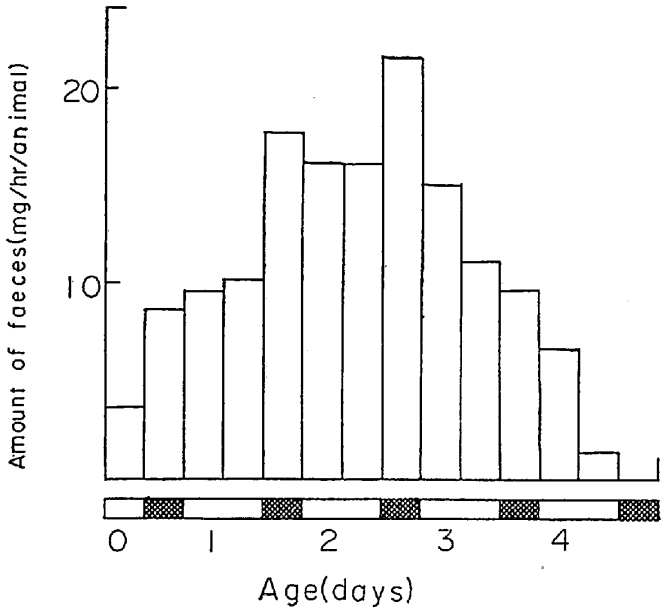

Fig. 8. Fluctuation of the amount of feces excreted. ▨: Dark period, □: light period.

last instar is shown in Fig. 7. The amount of food intake of the larvae increased with their development after ecdysis and it reached the highest level at the dark period of the 2nd and 3rd day. Then, it decreased gradually toward the gut-purging stage. The rate of food consumed was higher during the dark period than during the light period.

The amount of feces in a period from the 1st day to the 4th day in the last instar larvae paralleled the amount of food consumed during this same time interval. The amount of feces reached the highest level (22 mg/hr/larvae) during the dark period of the 3rd day, then decreased gradually toward the gut-purging stage and afterwards no excretion of feces was found. The excretion of feces at the 2nd day and the 3rd day showed a tendency to be larger during the dark period than the light period (Fig. 8).

From the results on the fluctuation in the amylase activities of the midgut tissue and lumen contents and in the feeding activity described above, it is clear that there is a cyclical change in feeding activity in the last instar larvae and the phase of the cyclical change of the amylase activity of midgut tissue is about 12 hr later than that of the feeding activity.

4. *Relationship between midgut amylase activity and cyclical change of feeding activity*

In the case of *ad libitum* feeding under normal photoperiod, activity of the midgut tissue amylase showed a rhythmical fluctuation and higher activity at 16: 00 on the 1st day and at 12: 00 on the 2nd day. In the same experimental condition, activity of the midgut content amylase showed a rhythmical fluctuation similar to

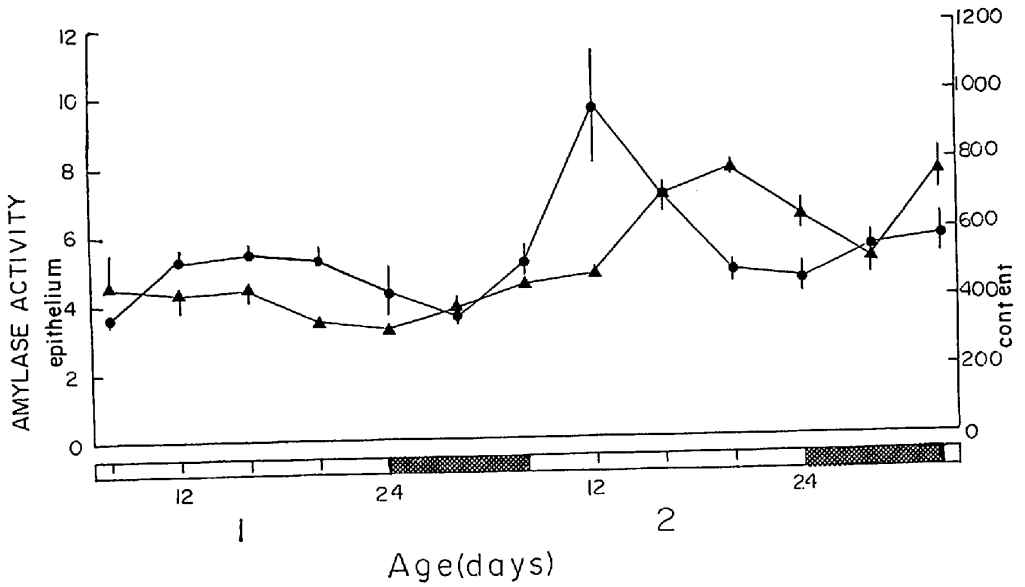

Fig. 9. Cyclical rhythm of the activities of two kinds of midgut amylase under *ad libitum* feeding and normal photoperiodism. ●: Midgut tissue amylase, ▲: midgut content amylase, ⧫ and ⧫: standard deviation of mean value of seven individuals as one group; ▨: dark period, □: light period.

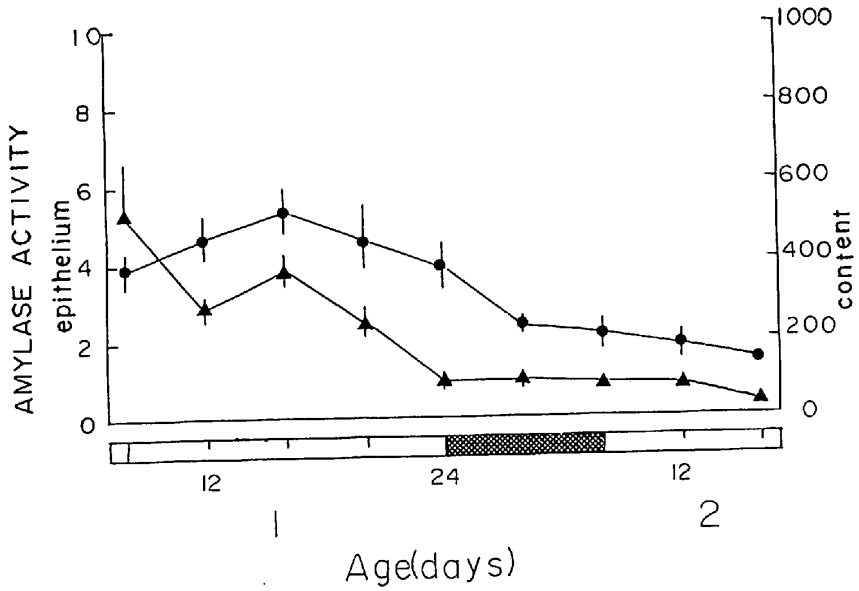

Fig. 10. Effect of starvation on the activity of two kinds of midgut amylases. ●: Midgut tissue, ▲: midgut contents, ⧫ and ⧫: standard deviation of mean value of seven individuals as one group; ▨: dark period, □: light period.

that of the midgut tissue amylase. On the 1st day, the amylase activity of midgut lumen contents changed a little being similar to that of the midgut tissue amylase, although the activity of the former was much higher than that of the latter. On the 2nd day, the activity of the midgut content amylase was the highest at 20: 00

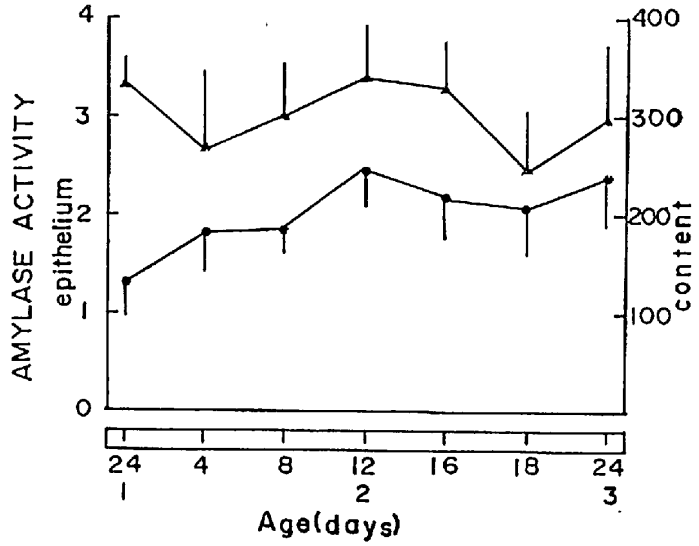

Fig. 11. Fluctuation of the activities of two kinds of midgut amylases under continuous light and *ad libitum* feeding. ●: Midgut tissue amylase, ▲: midgut content amylase, ⧫ and ⧫: standard deviation of seven individuals as one group. The larval age is the 2nd day of the 6th instar.

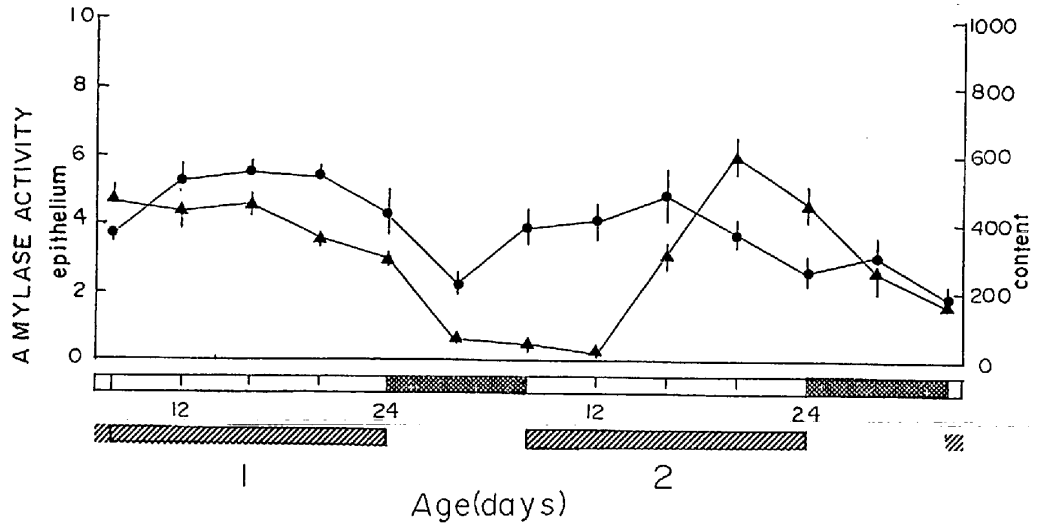

Fig. 12. Fluctuation of the activities of two kinds of midgut amylases under the starvation during the dark period and the feeding during the light period. ●: Midgut tissue amylase, ▲: midgut content amylase, ⧫ and ⧫: standard deviation; ▨: dark period, □: light period, ▨: feeding period.

This peak of amylase activity was 8 hr later than the peak of the amylase activity of midgut tissue (Fig. 9).

Under normal photoperiod and starvation, a fluctuation of the activities of two kinds of amylases were shown in Fig. 10. The midgut tissue amylase has

maintained high activity for 12 hr after starvation, then lowered gradually and did not show a peak of the activity, in the normal rearing condition. The activity in the midgut contents after starvation decreased more rapid than that of the midgut tissue amylase and was hardly detected at about 24 hr.

The amylase activities of the midgut tissue and the midgut contents, and the amounts of food intake and feces produced under continuous light indicated a slightly cyclical fluctuation (Fig. 11).

Amylase activities of larvae starved during the dark period under normal photoperiod, are given in Fig. 12. The activity of the midgut tissue amylase elevated gradually from 8: 00 to 16: 00 on the 2nd day, showed a peak value at 16: 00, and then lowered slowly. The highest value of the amylase activity in this case was one half as much as that in the condition of *ad libitum* feeding under the same photoperiod (Figs. 9 and 12). The peak of activity of the midgut tissue was earlier than that of the midgut lumen contents. In the case of the midgut content amylase, it decreased rapidly at the period from 0: 00 to 8: 00 on the 2nd day, increased from 12: 00 to 20: 00, reached the highest level at 20: 00, and then decreased gradually (Fig. 12).

In this experiment, the amylase activity of the midgut lumen contents under limited feeding condition showed larger fluctuation than that under the condition of *ad libitum* feeding. However, in the case of the midgut tissue amylase a reverse relationship between two feeding conditions was observed.

Discussion

In the present experiment, some enzymatic properties of amylases in the midgut tissue and lumen contents in larvae of the cabbage armyworm were determined. These values and those on digestive juice or its considerably purified amylase preparation and crude preparation of the midgut tissue in the silkworm are compared in Table 1. The optimal pH, optimal temperature and sensitivity of the digestive amylase for activation by addition of NaCl in these insects are similar. Since the Km value is higher in the digestive amylase of the silkworm than in that of the cabbage armyworm, the affinity of the digestive amylase of the latter for the substrate is probably higher. There were 4 electrophoretically active bands in the midgut tissue and lumen content amylases. These bands were located in the same positions. With considerably purified enzyme preparation of the midgut tissue or digestive juice from the 5th instar larvae of the silkworm, KURODA (1954) found three spots of amylases on the paper chromatogram, and NISHIDA & HAYASHIYA (1969) suggested that there are two types of amylases based on pH activity. Accordingly, these results seem to indicate that digestive amylases in moths consist of several isozymes.

In the present experiment, high amylase activities in the migdut tissue and lumen contents were observed in the first half of the last instar of the cabbage army-

Table 1. Comparison of enzymatic properties of the digestive amylase in the larvae of the cabbage armyworm, *Mamestra brassicae* L. and the silkworm, *Bombyx mori* L.

Insect	Optimal pH	Km mg/ml	Optimal temperature (°C)	Activation by NaCl	Number of electrophoretic bands or active spots	Direction of mobility
Cabbage armyworm, midgut wall	9.5	0.27	50	+ (10^{-3}~10^{-5} M)	4 (wall and contents)	→ +
Silkworm, midgut wall or digestive juice	9.2(d)^{H,Ka} 9.4(d)^{Ku}, 9.6(d)^{N}, 9.4–11.3(w)^{M}, 9.8(w)^{Ku}	0.42(d)^{Ka}	55(d)^{Ka}	+(2×10^{-2} M)(d)^{I}	2*(d)^{N,Ku}, 3*(w)^{Ku}	→ +(d)^{T}

(d): Digestive juice; (w): midgut wall; H: HORIE, 1959, with unpurified preparation; Ka: KANEKATSU, 1973, with purified preparation; Ku: KURODA, 1954, with partially purified preparation; N: NISHIDA & HAYASHIYA, 1969, with unpurified preparation; M: MORI, 1930, with partially purified preparation; I: ITO *et al.*, 1962, with unpurified preparation; T: TANAKA *et al.*, 1976, with unpurified preparation; *: active spots.

worm. The activity of the midgut content amylase was markedly higher than that of the midgut tissue amylase. Similar results have been obtained with the hawk moth, *Celerio euphorbiae* L. (HELLER & PIECHOWSKA, 1971). In the wax moth, *Galleria mellonella* L., the activity of gut amylase increased rapidly after the last larval ecdysis, reached the highest value in the 4-day-old and it decreased gradually until the pupal stage (KRIEG, 1972). Also, in the silkworm the activity of gut amylase and digestive juice amylase maintained a high level in the last instar (MATSUMURA, 1934). Accordingly, these results indicate that the fluctuation of gut amylase activity at larval stage varies considerably among species of the lepidopterous insects.

In the present experiment, the following relation between the midgut amylase activity and the rhythm of feeding activity was shown: i) The activities of the midgut tissue and content amylases during *ad libitum* feeding showed a cyclical rhythm, ii) the cyclical rhythm of amylase activities disappeared completely under a starvation treatment, iii) under continuous light the cyclical rhythm of feeding activity and digestive amylase activities was not markedly pronounced, and iv) under the condition of the feeding during the light period and the starvation during the dark period, the significant peak of amylase activity that appeared in the light period in a normal rearing condition, was not obtained. From these results, it is clear that activities of the midgut tissue and content amylases are probably induced through feeding activity. Furthermore, it can be claimed that cyclical rhythms of these amylases depend more on feeding rhythm than photoperiodism. Furthermore, a peak of the cyclical rhythm of the amylase activity of midgut tissue in the cabbage armyworm is some hours later than that of the feeding activity. Whether a cyclical change of the feeding activity and the midgut amylase activity in the present experiment is indicative of a circadian rhythm or not must analyze in future.

In the present experiment, the activity of the midgut content amylase was markedly more active than that of the midgut tissue amylase and the phase of a cyclical rhythm of the latter was slightly later than that of the former. Accordingly, it is suggested that the midgut content amylase originates from the midgut tissue amylase as a precursor. This speculation is supported by the findings of KURODA (1954) and KANEKATSU (1980) that digestive amylase is secreted into the lumen of the midgut through the peritrophic membrane from cells of the midgut tissue in the larvae of the silkworm.

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