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## Comparison of biological and biochemical parameters of eri-silkworms, *Samia cynthia ricini* (Lepidoptera: Saturniidae), reared on artificial and natural diets

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### Abstract

In the present study, eri-silkworms were reared on cassava leaves (its natural host plant) and Silkmate L4M from 1<sup>st</sup> to 5<sup>th</sup> instars. The lengths of the larval and pupal periods of eri-silkworms reared on the artificial diet were extended compared to those reared on cassava leaves. Comparison between the larval and pupal weights showed that eri-silkworms reared on the artificial diet had higher larval and pupal weights than those reared on cassava leaves, but the cocoon shell weight did not differ between the groups. In addition, total hemocyte count, total hemolymph protein concentration, total lipid concentration, and  $\alpha$ -amylase activity in eri-silkworms reared on Silkmate L4M were lower than those in eri-silkworms reared on cassava leaves. Results indicated that the artificial diet containing mulberry leaves affected many biological and biochemical parameters of the eri-silkworm compared to rearing on cassava leaves.

**Keywords:** Eri-silkworm, *Samia cynthia ricini*, saturniidae, artificial diet

### 1. Introduction

The eri-silkworm, *Samia cynthia ricini* (Saturniidae), is a commercial silk-producing insect that is now reared year round in many countries with the expectation of its being utilized for diverse purposes [1]. It is one of the most exploited, domesticated, and commercialized non-mulberry silkworms. The eri-silkworm produces many generations per year [2], and the larvae feed on several host plant species, including castor oil plants, aianthus, cassava, kesseru, and plumeria. Moreover, larvae of the eri-silkworm will eventually eat any kind of plant leaves, unless the leaves are too hard or hairy. They also eat artificial diets containing extracts from various plants, and may subsequently show symptoms, such as death from poisoning and growth inhibition, in response to particular plants. For this reason, eri-silkworm larvae have been successfully used in bioassays and analyses to evaluate the defense activities and defense levels of plants against herbivorous insects [3]. In addition, the eri-silkworm was also used as an insect model to study innate immune response and antibacterial activity [4-11].

Since a commercial diet for "polyphagous" mutant silkworms, Silkmate L4M, which contains a low percentage of mulberry leaf powder (4%), became commercially available [12], it has been used to rear several lepidopteran insects, including the eri-silkworm [7, 9-11, 13]. However, Konno *et al.* [14] reported that mulberry leaves are highly toxic to generalist caterpillars that do not feed on mulberry trees as host plants, such as the eri-silkworm and cabbage moth *Mamestra brassicae* Linnaeus (Noctuidae) due to the latex ingredients exuded from damaged leaf veins. Furthermore, sugar-mimicking alkaloids in mulberry latex are toxic to eri-silkworm larvae by the inhibition of midgut sucrase and trehalase activity [3].

Silkmate L4M has been used to rear eri-silkworms in the laboratory as an insect model in physiological and immunological studies [7, 9-11]. Based on the above-mentioned reports, mulberry leaf latex has toxic effects on the eri-silkworm. Hence, the aim of this study was to consider whether Silkmate L4M is suitable for rearing eri-silkworms. In this study, we observed larval body weight, length and weight of the silk gland, weights of pupae and shells, total hemocyte count, total hemolymph protein concentration in larvae, total lipid concentration in pupae, and  $\alpha$ -amylase activity in the gut of eri-silkworms reared on cassava leaves or Silkmate L4M. Understanding the nutritional physiology of eri-silkworms when reared on an artificial diet and determining the factors affecting its life history could help in planning effective rearing options for this insect.

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## 2. Materials and Methods

### 2.1 Insect rearing

The white plain eri-silkworm breed, *S. cynthia ricini*, was used for this experiment. Larvae were reared separately under laboratory conditions at  $25 \pm 2$  °C with a relative humidity of  $65 \pm 5\%$ . Newly hatched larvae ( $n = 30$ ) were randomly selected and individually transferred with the help of a small paint brush to the rearing trays. They were reared on either a commercial artificial diet (Silkmate L4M) or fresh cassava leaves from hatching to the 5<sup>th</sup> instar. The treatments were replicated three times. For larvae reared on cassava leaves, 1<sup>st</sup> - and 2<sup>nd</sup> -instar larvae were fed tender leaves cut into small pieces; medium-aged leaves were fed to 3<sup>rd</sup> -instar larvae, and mature leaves to 4<sup>th</sup> -and 5<sup>th</sup> -instar larvae. For larvae reared on the artificial diet, food was cut into small pieces and fed to 1<sup>st</sup> -and 2<sup>nd</sup> -instar larvae. Larger pieces were fed to 3<sup>rd</sup> - to 5<sup>th</sup> -instar larvae. The larvae were fed four times per day except during molting periods. The quantity of food was increased with larval age to fulfill their nutritional requirements. Beds were cleaned regularly, and mature larvae were transferred to suitable cages to spin cocoons [15].

### 2.2 Artificial diet

Silkmate L4M (a diet for “polyphagous” mutants of the silkworm, mulberry powder content: 4%) was purchased from Nihon Nosan Kogyo Co. (Yokohama, Japan). First, 250 g of Silkmate L4M was weighed in a stainless steel container ( $14 \times 20 \times 7.5$  cm). After the addition of 750 ml of water and thorough mixing, the food was steamed for 40 min. The food was mixed thoroughly again, and then kept in a refrigerator until use [12].

### 2.3 Larval and pupal duration

The larval duration, defined as the period between the hatching of eggs and maturity, was recorded under each treatment in days. The pupal duration was defined as the period from the onset of pupariation until the emergence of the adult.

### 2.4 Determination of the weight of the larva, pupa, and pupal shell

The weight of the larva was measured every day. Ten larvae from each treatment were randomly selected and weighed, and the average was calculated. For single shell weight, cocoons were cut open to remove the pupae, and the weight of the shell was recorded separately.

### 2.5 Measurement of the length and weight of silk glands

On day 3 of the 5<sup>th</sup> instar, three larvae were collected from each group. The larvae were dissected to collect the silk glands. The silk glands were washed in ice cold 20 mM phosphate buffer, pH 6.0, and blotted on a filter paper. Silk glands were divided into three parts, the anterior silk gland (ASG), middle silk gland (MSG), and posterior silk gland (PSG), and their lengths and weights were measured.

### 2.6 Determination of hatchability

Three pairs of 2-day-old adults from larvae reared on both diets were allowed to mate in a screen cage ( $25 \times 25 \times 25$  cm) for 2 days, and then female moths were individually housed in a screen cage to allow them to lay eggs. Three days later, the number of eggs laid on the surface of the cage was counted. The hatchability of eggs was determined 10 days after the eggs were laid.

### 2.7 Determination of total hemocyte count

Hemolymph was obtained from 3-day-old 5<sup>th</sup> -instar larvae by cutting their prolegs with microscissors. Hemocyte counts were performed on individual larvae. The total hemocyte count (THCs) was obtained by applying diluted hemolymph (300  $\mu$ l hemolymph mixed with 300  $\mu$ l 20 mM phosphate buffer, pH 6.0, containing 0.37%  $\beta$ -mercaptoethanol) to a Neubauer hemocytometer. THC was expressed as number of cells per ml of hemolymph.

### 2.8 Determination of the total protein concentration

Hemolymph samples were collected from 3-day-old 5<sup>th</sup> -instar larvae by making an incision on their prolegs with microscissors. The hemolymph was transferred into a microtube immersed in ice. Then, 50  $\mu$ l of hemolymph was diluted in 300  $\mu$ l of 20 mM phosphate buffer, pH 6.0, containing a small amount of phenylthiourea (Nacalai tesque, Kyoto, Japan). Hemolymph samples were centrifuged at 5,000 g at 4 °C for 5 min, and an aliquot (20  $\mu$ l) was used for protein determination. The protein concentration in the hemolymph was determined using a Bio-Rad protein assay kit with bovine serum albumin as the standard protein (Bio-Rad Laboratories, Richmond, CA, USA).

### 2.9 Determination of total lipid concentration

Lipids were extracted and quantified using chloroform and methanol following Folch *et al.* [16] as revised by Post *et al.* [17]. Briefly, three female pupae developed from larvae reared on each diet were weighed individually, and 20 pupal body-weight volumes of a 2:1 chloroform/methanol mixture were added, followed by homogenization. The homogenate was transferred to a flask and shaken vigorously at room temperature for 20 min. The entire volume was filtered through a No. 1 Whatman filter paper. The filtrate was mixed with 0.2 volumes of 0.9% NaCl and allowed to separate into two phases. The upper phase was discarded, and the lower phase was transferred into a pre-weighed aluminum dish. The contents were evaporated at 60 °C until dry. The lipid remaining in the aluminum dish was weighed and the result was considered to represent the mass of lipids per pupa.

### 2.10 Determination of $\alpha$ -amylase activity

The midgut was collected from 3-day-old 5<sup>th</sup> -instar larvae. Samples were washed in ice-cold 20 mM phosphate buffer, pH 6.0, and blotted on a filter paper. Individual midguts were homogenized in 500  $\mu$ l of 20 mM phosphate buffer, pH 6.0. The homogenates were centrifuged at 12,000 g at 4 °C for 10 min. The supernatant was kept at -20 °C until use as an enzyme source. The amount of protein was determined prior to the  $\alpha$ -amylase assay using a protein dye-binding method (Biorad, Hercules, CA, USA), with bovine serum albumin as the standard.  $\alpha$ -Amylase activity was determined using a modified version of the procedures reported by Rekha *et al.* [18]. The activity was determined by incubating 40  $\mu$ l of midgut extract with 40  $\mu$ l of 0.2% starch solution (Sigma, St. Louis, MO, USA) in 20  $\mu$ M phosphate buffer, pH 6.0, at 37 °C for 1 h. The reaction was stopped by the addition of 20  $\mu$ l of 1 M HCl, and the amount of residual starch was estimated using 100  $\mu$ l of iodine solution (0.5% I<sub>2</sub> and 5% KI). The reaction volume was adjusted to 1,000  $\mu$ l by the addition of 800  $\mu$ l of distilled water, and the absorbance at 580 nm was measured using a spectrophotometer (Jenway, Stone, Staffordshire, UK).  $\alpha$ -Amylase activity was expressed as  $\mu$ g of starch hydrolyzed/ $\mu$ g of protein/h.

### 2.11 Statistical analysis

Differences in the developmental period, length of silk glands, weight of silk glands, weight of pupa, weight of shell, hatchability, THC, total hemolymph protein concentration, total lipid concentration, and  $\alpha$ -amylase activity were tested using Student's t-test ( $P < 0.05$ ) in the SPSS program, version 11.0.

## 3. Results

### 3.1 Developmental period

The lengths of 1<sup>st</sup> to 4<sup>th</sup> larval instar periods did not significantly differ between larvae reared on cassava leaves and those on the artificial diet. The length of the 5<sup>th</sup> larval instar and pupal periods of insects reared on the artificial diet were 3 days longer than that of insects reared on cassava leaves (Table 1). In addition, the hatchability of the F1 generation of insects fed on both cassava leaves and artificial diet was high ( $91.98 \pm 6.58\%$  and  $93.12 \pm 7.21\%$ , respectively), but not significantly different.

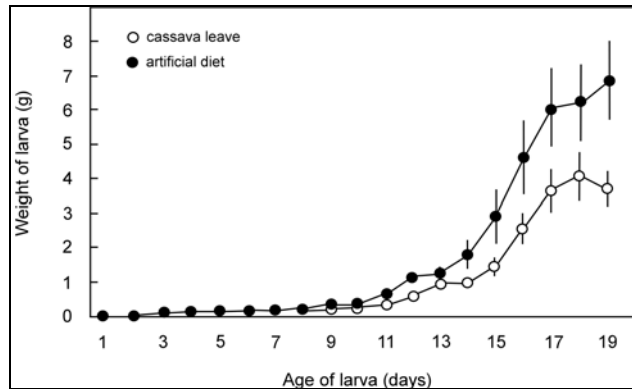
**Table 1:** Developmental period of larvae and pupae after feeding with cassava leaves and artificial diet.

Type of diet	Larva (days)					Pupa (days)	Total (days)
	1 <sup>st</sup> instar	2 <sup>nd</sup> instar	3 <sup>rd</sup> instar	4 <sup>th</sup> instar	5 <sup>th</sup> instar		
Cassava leaves	3.5 $\pm 0.51$	3.60 $\pm 0.50$	3.43 $\pm 0.50$	3.73 $\pm 0.45$	5.00	13.50 $\pm 0.51$	32.76 $\pm 1.81$
Artificial diet	3.53 $\pm 0.50$	3.63 $\pm 0.49$	3.47 $\pm 0.51$	3.83 $\pm 0.38$	5.63 $\pm 0.49^*$	15.57 $\pm 0.50^*$	35.67 $\pm 1.77^*$

Asterisks above the means within each stage indicate significant differences ( $P < 0.05$ ) between insect reared on cassava leaves and insect reared on artificial diet.

### 3.2 Larval weight

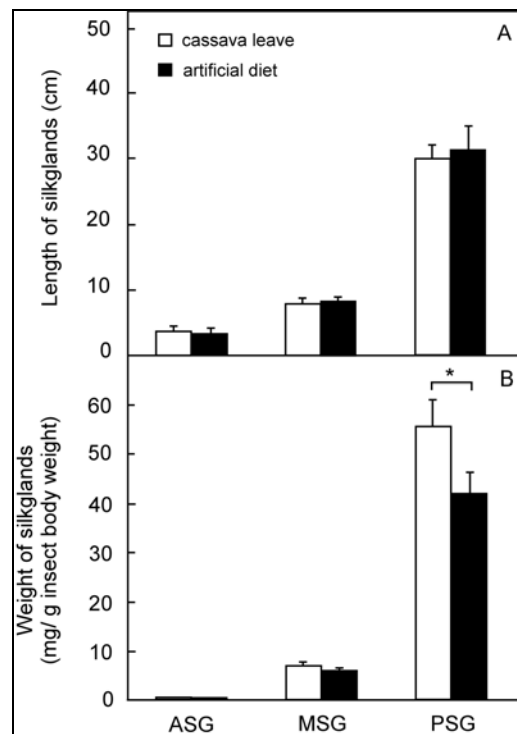
After weighing 1<sup>st</sup>- to 5<sup>th</sup>-instar larvae, we found no differences between larvae reared on cassava leaves and artificial diet from 1 to 8 days after hatching. After day 9, weights of larvae reared on both diets increased dramatically. The body weight of larvae reared on the artificial diet was higher than that of larvae reared on cassava leaves from day 14 after hatching. The larvae weighed the most on day 19,  $6.86 \pm 1.16$  g and  $3.66 \pm 0.52$  g for larvae reared on the artificial diet and cassava leaves, respectively (Fig. 1).



**Fig 1:** Larval weights of eri-silkworms, *S. cynthia ricini*, fed on cassava leaves and on the artificial diet. Bars indicate the means of three independent biological replicates with standard deviations (SDs).

### 3.3 Length and weight of silk glands

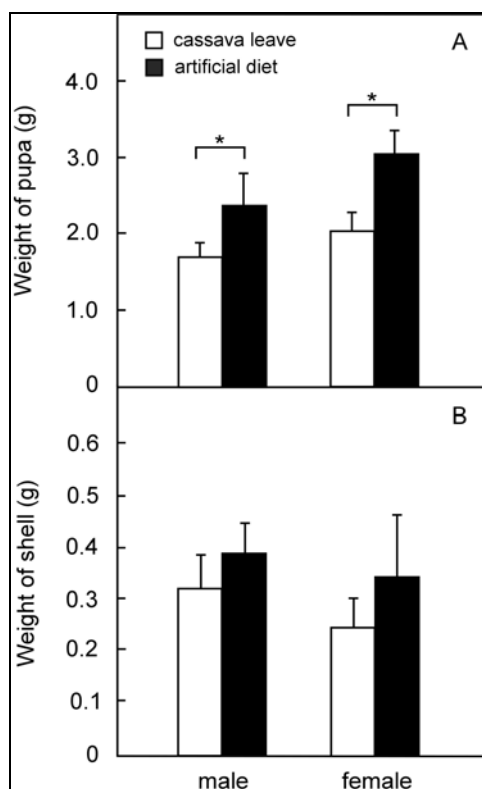
The lengths of all three parts of the silk gland, ASG, MSG and PSG, did not differ between larvae reared on cassava leaves and larvae reared on the artificial diet (Fig. 2A). ASG and MSG weights in larvae reared on both diets did not differ, but PSG in larvae reared on cassava leaves was 13.38 mg heavier than that of larvae reared on the artificial diet ( $P < 0.05$ ) (Fig. 2B).



**Fig 2:** Silk gland lengths (A) and weights (B) from larvae of eri-silkworms, *S. cynthia ricini*, fed on cassava leaves and the artificial diet. Bars indicate the means of three independent biological replicates with standard deviations (SDs). Asterisks above the bars within each part of silk gland indicate significant differences ( $P < 0.05$ ) between eri-silkworms fed on cassava leaves and those fed the artificial diet.

### 3.4 Weight of pupa and shell

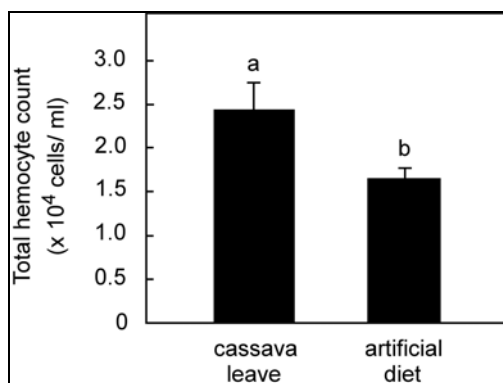
The weights of male and female pupae developed from larvae reared on artificial diet were  $2.28 \pm 0.4$  g and  $2.92 \pm 0.32$  g, respectively, whereas the weights of male and female pupae developed from larvae reared on cassava leaves were  $1.69 \pm 0.17$  g and  $1.99 \pm 0.25$  g, respectively. This result clearly shows that pupae in the artificial diet fed group were significantly heavier than pupae in the cassava leaves group ( $P < 0.05$ ) (Fig. 3A). On the other hand, the weights of the shell in both males and females did not differ between insects fed on cassava leaves and those on the artificial diet ( $P > 0.05$ ) (Fig. 3B).



**Fig 3:** Pupa (A) and shell (B) weights of eri-silkworms, *S. cynthia ricini*, fed on cassava leaves and the artificial diet. Bars indicate the means of three independent biological replicates with standard deviations (SDs). Asterisks above the bars within each sex indicate significant differences ( $P < 0.05$ ) between eri-silkworms fed on cassava leaves and those fed the artificial diet.

### 3.5 Total hemocyte count

The total hemocyte count in 3-day-old 5<sup>th</sup>-instar larvae reared on cassava leaves was significantly higher than that of larvae reared on artificial diet ( $P < 0.05$ ). The total hemocyte count in the cassava leaves-fed group was  $2.45 \pm 0.33 \times 10^4$  cells/ml, while the total hemocyte count in the artificial diet-fed group was only  $1.61 \pm 0.12 \times 10^4$  cells/ml (Fig. 4).

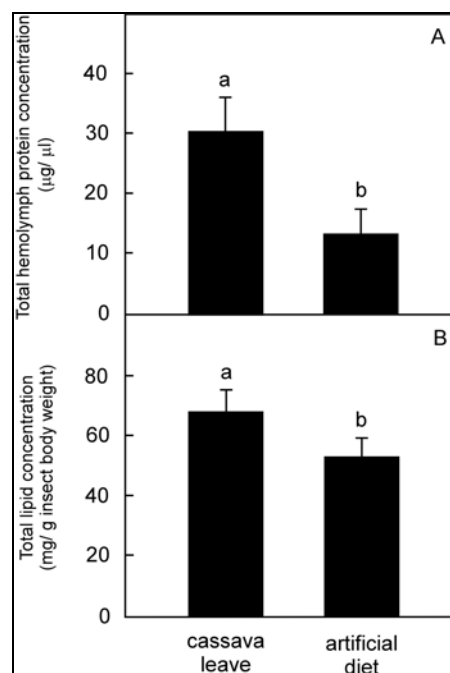


**Fig 4:** Total hemocyte counts from larvae of eri-silkworms, *S. cynthia ricini*, fed on cassava leaves and the artificial diet. Bars indicate the means of three independent biological replicates with standard deviations (SDs). Different letters above the bars indicate significant differences ( $P < 0.05$ ) between eri-silkworms fed on cassava leaves and those fed the artificial diet.

### 3.6 Total hemolymph protein concentration and total lipid concentration

The total hemolymph protein concentration in 3-day-old 5<sup>th</sup>-

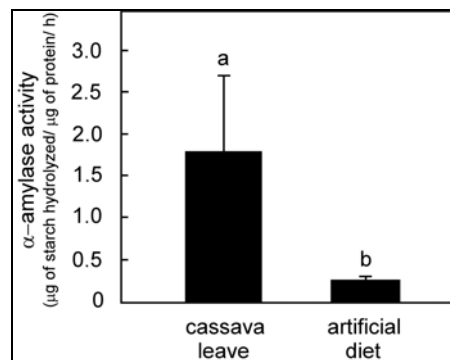
instar larvae reared on cassava leaves was  $30.27 \pm 5.87 \mu\text{g}/\mu\text{l}$ , which was significantly higher than that in larvae reared on the artificial diet ( $13.24 \pm 3.92 \mu\text{g}/\mu\text{l}$ ) ( $P < 0.05$ ) (Fig. 5A). Similarly, the total lipid concentration in pupae developed from larvae reared on cassava leaves was significantly higher than that in pupae developed from larvae reared on the artificial diet ( $67.73 \pm 6.88 \text{ mg/g}$  and  $52.13 \pm 6.30 \text{ mg/g}$  insect body weight, respectively) ( $P < 0.05$ ) (Fig. 5B).



**Fig 5:** Total hemolymph protein concentration in larvae (A) and total lipid concentration in pupae (B) of eri-silkworms, *S. cynthia ricini*, fed on cassava leaves and the artificial diet. Bars indicate the means of three independent biological replicates with standard deviations (SDs). Different letters above the bars indicate significant differences ( $P < 0.05$ ) between eri-silkworms fed cassava leaves and those fed the artificial diet.

### 3.7 $\alpha$ -Amylase activity

The activity of  $\alpha$ -amylase in the gut of larvae reared on cassava leaves was higher than that in those reared on the artificial diet ( $1.77 \pm 0.92 \mu\text{g}$  and  $0.25 \pm 0.03 \mu\text{g}$  of starch hydrolyzed/ $\mu\text{g}$  of protein/h, respectively) (Fig. 6).



**Fig 6:**  $\alpha$ -Amylase activity in the gut of eri-silkworm larvae, *S. cynthia ricini*, fed on cassava leaves and the artificial diet. Bars indicate the means of three independent biological replicates with standard deviations (SDs). Different letters above the bars indicate significant differences ( $P < 0.05$ ) between eri-silkworms fed on cassava leaves and those fed the artificial diet.

#### 4. Discussion

Although an artificial diet can obviate the serious drawbacks of host plant leaves, such as seasonal limitation on supply, possible harm from parasites or pesticides, and high labor cost, insects reared on the artificial diet during all instars were of lower quality than those fed on fresh host plant leaves, which is reflected in many measures, such as the filament quality of cocoons, survival rate of young larvae, and resistance to bacterial and viral diseases [19-21]. The results obtained in this study using the eri-silkworm, *S. cynthia ricini*, as a model showed that an artificial diet affected the lengths of the larval and pupal periods, larval weight, pupal weight, weight of PSG, total hemocyte count, total hemolymph protein concentration, total lipid concentration, and  $\alpha$ -amylase activity. Hatchability, the lengths of ASG and MSG, and shell weight did not differ between insects reared on the artificial diet and cassava leaves.

Because Silkmate L4M contains a mulberry leaf powder, this diet must also contain dried mulberry latex. Mulberry latex contains very high concentrations of sugar-mimicking alkaloids (alkaloidal glycosidase inhibitors, polyhydroxy alkaloids, and iminosugars), such as 1, 4-dideoxy-1, 4-imino-D-arabinitol (D-AB1), 1-deoxynojirimycin (DNJ), and 1, 4-dideoxy-1, 4-imino-D-ribitol, as defense molecules against herbivorous insects, together with other high-molecular-weight defense molecules [14]. In *Morus australis*, a wild mulberry native to mainland Japan and Okinawa Island, sugar-mimicking alkaloids comprise a total of 8–18% of the dried latex. This amount of dried latex may be high enough to interfere with the growth and some biological parameters of the eri-silkworm. Our results are similar to the report that mulberry leaves (*Morus* spp.) are highly toxic to generalist caterpillars that do not feed on mulberry trees as host plants, such as the cabbage moth *M. brassicae*, due to the ingredients in the latex exuded from damaged leaf veins [14]. Compared to *Bombyx mori*, the eri-silkworm was approximately  $10^4$  times more sensitive to D-AB1 [3].

In *B. mori*, individuals reared on an artificial diet during all instars were also not as high quality as were those fed on fresh mulberry leaves, which is reflected in many characteristics, especially resistance to bacterial and viral diseases [19-21]. Comparison of the total hemocyte count between *S. cynthia ricini* larvae reared on cassava leaves and those fed the artificial diet, showed that total hemocyte count in larvae reared on cassava leaves was significantly higher than that in larvae reared on the artificial diet, by about 1.52-fold. The present study results indicate that the immune system may be affected by the artificial diet containing mulberry leaves, which is toxic to other insects that do not feed on mulberry leaves as their host plant. In addition to its effects on many biological parameters of the eri-silkworm, some biochemical parameters were also affected by the artificial diet, including total hemolymph protein concentration in larvae, total lipid concentration in pupae, and activity of  $\alpha$ -amylase in larvae. Eri-silkworms reared on the artificial diet had lower total protein concentration in larvae,  $\alpha$ -amylase activity in larvae, and total lipid concentration in pupae compared to insects reared on cassava leaves.

Because  $\alpha$ -amylase is an important class of digestive enzyme, many insect species depend on the effectiveness of their amylases for survival. Studies of insect amylase activity can help to clarify the compatibility of a diet with insect development [22]. We therefore focused our attention on the effects of the artificial diet on  $\alpha$ -amylase activity in the eri-silkworm. In this study,  $\alpha$ -amylase activity in the gut of

larvae fed the artificial diet was lower than that in larvae fed on cassava leaves by approximately seven fold. This phenomenon also supports the toxicity of sugar-mimicking alkaloids in the artificial diet. Sugar-mimicking alkaloids are analogs of sugar molecules that inhibit many enzymes related to sugar metabolism, and they are found in various plants and microorganisms [23, 24]. Rearing the eri-silkworm using an artificial diet containing mulberry leaves may not be a suitable choice, and the development of an artificial diet containing their natural host plants, such as cassava leaves or castor leaves, that does not have an effect on the biological and biochemical properties of the eri-silkworm is needed for best silkworm quality.

#### 5. Conclusion

Silkmate L4M contains mulberry leaf powder which is toxic to eri-silkworm, *S. cynthia ricini*. This diet prolonged the larval and pupal periods and affected various biochemical parameters by reducing the total hemocyte count, total hemolymph protein concentration, total lipid concentration and  $\alpha$ -amylase activity. The suitable artificial diet for rearing the eri-silkworm in the laboratory scale should be selected carefully based on their effects on biological and biochemical parameters.

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