Original Article

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A simple artificial diet available for research of silkworm disease models

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SUMMARY This study was performed with the aim of making a very simple recipe of silkworm diet for research purposes, especially screening of drug candidates. We prepared a diet containing mulberry leaves powder and soybean flour at different ratios, fed them to fifth instar silkworm larvae, and observed their growth. We selected the diet with 1:1 ratio of mulberry powder and soybean flour, named MS-11, and used for further experiments. MS-11 diet was available for oral administration of drugs in silkworm hyperglycemic model and infection model. The availability of a simple artificial diet for experiments that require feeding silkworms will enhance the use of silkworms for biological, biotechnological, and pharmacological researches.

Keywords Silkworm, artificial diet, mulberry, research

1. Introduction

Silkworm (*Bombyx mori*) is a lepidopteran invertebrate that has been domesticated for silk production with a long history of breeding (*1*). Apart from using them for silk, silkworm larvae have gained attention as animal model of various human diseases. The basic common pathways shared by silkworms with mammals, their body features and size and ease in handling, and less ethical concerns make silkworms appropriate for research purposes before moving on to higher animal models (*2*).

The fact that human pathogenic microorganisms kill silkworms and clinically used antibiotics cure those infections with comparable effective dose fifty (ED_{50}) values (*3*) to those of mammals make silkworm suitable for using as an infection model. The activation of innate immunity and response towards infection (*4*) makes them suitable for the study of host-pathogen interactions. Further, similarity in pharmacokinetics (*5,6*) and toxicities (*7*) of compounds make them more reliable and appropriate for using them to identify lead molecules that have therapeutic effect and a potential to further towards clinical applications.

Silkworms have been applied for the screening of novel antimicrobial agents with novel mechanism of actions from natural product (*8*) and chemical libraries (*9-11*), for screening of novel virulence factors of pathogenic microorganisms (*12,13*). The elevation of glucose in silkworm blood after ingestion of glucose and sucrose diet and lowering of this level by clinically used anti-diabetic drugs (*14*) makes them suitable for screening of compounds that may be applied as anti-diabetes agents. The fate of compounds after ingestion such as bioavailability, absorption, distribution, metabolism, and excretion can be studied as silkworms have basic metabolic pathway common to mammals with involvement of cytochrome P450 enzyme for metabolism (*5*). On the basis of pharmacokinetic parameters, therapeutic activities of compounds with similar *in vitro* activities could be differentiated by evaluation with silkworms (*11*). Therefore, silkworm larvae are appropriate for pharmacological researches.

Usually, silkworms are reared on mulberry leaves as they have a selective preference over it. As mulberry leaves are not available all year round, several artificial diets for silkworm rearing are commercially available. There have been several studies to establish artificial diets for silkworms focusing on improving the silk production (*15-17*). However, diets that can be easily prepared and suitable for establishing silkworms as disease models are lacking. In our laboratory, we have been routinely using artificial diet for research purposes. The comparison of the artificial diet with mulberry leaves on infection model of silkworm showed that the killing ability of a Gram-positive bacteria, *Staphylococcus aureus*, was same regardless of the diet fed (*18*).

Even when mulberry leaves are available for silkworm rearing, some disease models require the use of artificial diets, especially in conditions where silkworms must be fed with substances under study.

For instance, when screening for blood glucose level lowering agents, silkworms should be fed high glucose/sucrose diet, when determining oral toxicity of compounds, the compounds must be fed to silkworms. In such experiments, it is necessary that silkworms feed on the substances and this in only possible when artificial diet is available.

In this study, we established a simple artificial diet, MS-11, for pharmacological and biological experiments requiring feeding silkworms. The establishment of such diet will help researchers around the world to prepare their own artificial diet in the laboratories and perform desired researches. This will expand the use of silkworms in research which is limited to the laboratories where rearing silkworms in artificial diets is a common practice.

2. Materials and Methods

2.1. Preparation of artificial diet

Artificial diets were prepared by mixing five different proportions of mulberry leaves powder (Healthy Company, Japan) and soybean flour, and agar (Nacalai, Japan) in 100 mL water (Figure 1A). They were autoclaved at 121°C for 15 minutes, mixed and kept at 4°C for use. Ready to use artificial diet, Silkmate 2S diet, was purchased from Nosan Corporation, Japan.

2.2. Rearing of silkworms

Silkworms were reared according to previously described methods (*8,10*). Briefly, hatched larvae from silkworm eggs (Hu·Yo × Tukuba·Ne; Ehime Sanshu), were fed artificial diet, SilkMate 2S (Nihon Nosan Co., Ltd., Kanagawa, Japan) until 4th or 5th instar larvae as mentioned. Silkworms were maintained at 27°C all the time.

2.3. Measurement of glucose level in silkworm hemolymph

The 4th instar larvae were fed Silkmate diet or MS-11 diet until they become 5th instar larvae. The 5th instar larvae weighing between 0.9 to 1.1 g were starved overnight. Next day, the larvae were fed respective diets with or without 10% sucrose (w/w) (Fujifilm wako, Japan). Acarbose (80 mg/g, LKT laboratories Inc., USA), a known clinically used antidiabetic drug, was mixed with the 10% sucrose containing diets as a positive control. After 1 h of feeding, hemolymph was collected by cutting the abdominal prolegs, and glucose level was measured using a glucometer (Accu-Chek: Roche, Basel, Switzerland).

2.4. Determination of oral therapeutic activity of antibiotics

The 5th instar silkworm larvae were fed overnight with MS-11 diet. *Staphylococcus aureus* MSSA1 was grown in sheep blood agar plate at 37°C overnight, inoculated in tryptic soy broth, and grown at 37°C with shaking at 200 rpm for 18 hours. The full growth was 10-fold diluted with 0.9% saline, 50 µL of which was injected into the hemolymph of each silkworm larva. The infected larvae were fed with MS-11 diet containing various concentrations of chloramphenicol or tetracycline. The survival was observed and recorded for each dose when all the silkworms fed with no antibiotic diet died. ED_{50} values were calculated by logistic regression analysis using the logit link function.

3. Results

3.1. Growth of silkworms depends upon the component of artificial diet

We prepared five different artificial diets with varying proportions of mulberry leaves powder and soybean flour (Figure 1A). We fed 1 g of these diets to each of the 5th instar first day silkworm larvae and observed their growth. We found that the larvae preferred the diets containing mulberry leaves powder. Among the

A.

Diet	Ingredients per 100 mL water		
	Mulberry powder	Soybean flour	Agar
A	20 g	0 g	2.5 g
B	16 g	4 g	2.5 g
C	10 g	10 g	2.5 g
D	4 g	16 g	2.5 g
E	0 g	20 g	2.5 g
F	Silkmate diet		

B.

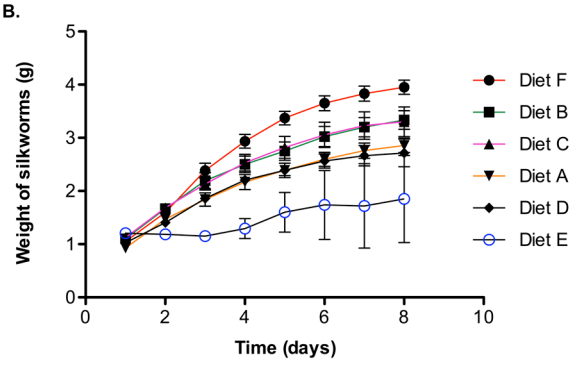

Figure 1. Silkworm artificial diet and growth of silkworm larvae. (A) Composition of the artificial diets used in this study. Diets were prepared as mentioned in Materials and Methods section. **(B)** Growth of the artificial diet fed silkworm larvae. One gram of each diet was fed per larva per day and remaining diets were removed each day. Growth of each larva was recorded each day for 8 days and data is shown as mean ± SEM.

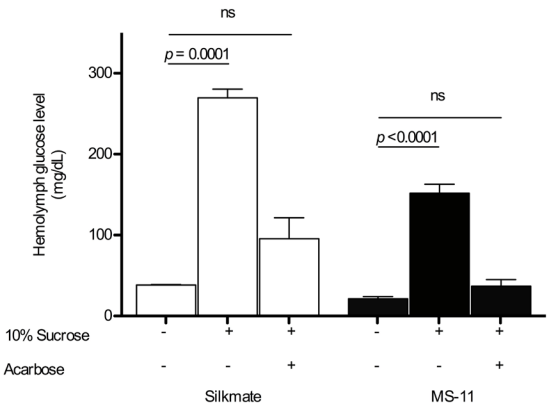

Figure 2. Hemolymph glucose level of silkworm larvae. Larvae were fed with 10% sucrose containing Silkmate or MS-11 diet with or without acarbose (80 mg/g). After one hour, glucose level in hemolymph was measured by a glucometer. Data is shown as mean ± SEM and analyzed by one-way analysis of variance (AVOVA) with Dunnett's multiple comparison test compared with no sucrose diet, ns: not significant.

five diets tested, the most weight gain was observed in silkworms fed with diet B and C, however, the growth of all the five diets fed silkworms was smaller than that of the commercially available diet F (Silkmate) fed silkworms (Figure 1B). Although the larvae grew normally until day 8, they could not form cocoon, suggesting that some essential components for cocoon formation are lacking on the diets.

3.2. Glucose lowering activity of an orally administered antidiabetic drug

We selected diet C (mulberry powder:soybean flour,1:1), named as MS-11, for further experiments. We used MS-11 to check the hemolymph glucose level in silkworm by mixing 10% sucrose in the diet and compared with the Silkmate diet. We found that like Silkmate diet, MS-11 diet containing 10% sucrose fed silkworm hemolymph showed higher glucose levels, which was lowered by a clinically used anti-diabetic drug acarbose (Figure 2). The overall high hemolymph glucose level in Silkmate diet-fed silkworms may be due to the sugar content in the diet itself. These results suggested that MS-11 diet containing 10% sucrose could be used for the glucose level lowering experiments and is suitable for screening for identification of blood-glucose-lowering agents.

3.3. Oral administration of antibiotics by using artificial diet in a silkworm infection model

To assess oral therapeutic effectiveness of antibiotics, we injected silkworms with a clinical isolate of methicillin-sensitive strain of *S. aureus*, MSSA1 into the hemolymph. We then mixed different concentrations of clinically used antibiotics chloramphenicol and tetracycline with MS-11 diet and fed the infected

Table 1. Therapeutic activity of antibiotics by oral administration

Antibiotic	ED_{50} value (μg/g larva)	
	MS-11 diet	Silkmate diet*
Chloramphenicol	41	40
Tetracycline	9	8

*ED_{50} values were previously reported (*3*).

silkworms. We counted the number of the survival of silkworms and calculated the ED_{50} values of the antibiotics. We found that the ED_{50} values of the antibiotics per oral route to be consistent with those previously obtained using the Silkmate diet (Table 1). These results suggest that MS-11 diet prepared in this study is available for determining therapeutic activities of orally effective antibiotics.

4. Discussion

Several studies have been performed to assess the components essential for the growth of silkworm and for optimum silk production by using artificial diet (*19*). Comparison between natural and artificial diets fed silkworms have also been done that analyzed gut microbiota (*20*) as well as proteomic (*21*) and metabolomic (*22*) profiles. Most of the studies on artificial diet have mainly focused on silk production, so far. In the recent scenario of growing interest of using silkworms as drug discovery models, artificial diet that is simple, can be widely used, and applicable for research purposes is desirable.

Silkworms sense the odor of mulberry and are attracted by β-γ-hexenol and α-β-hexenal components in the mulberry (*23*), which is sensed by *GR66* gene encoding a putative bitter gustatory receptor (*24*) that are responsible for the mulberry-specific feeding preference of the silkworms. As expected, we found that the larvae preferred diet that contained mulberry powder. Of note, the MS-11 diet is for research and not for silkworm rearing purpose. Silkworms can be reared in mulberry leaves and MS-11 diet can then be used for subsequent feeding experiments. MS-11 diet is simple with only two major ingredients and can be easily prepared in individual laboratories. Besides, this composition ensures that there is less interference of the diet components to the experiments. The diet is applicable to experiments that require feeding compounds/extracts to silkworms to assess various biological activities through oral route of administration. Here we showed two such examples; assessing blood glucose-lowering activity and oral antimicrobial activity of clinically used drugs. MS-11 diet can be utilized for other similar experiments including but not limited to assessing oral toxicity, bioavailability, absorption, digestion/metabolism of compounds, and effect of compounds including food

products, probiotics in immunity, and application in food/nutrition science.

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