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Accumulation of Dietary Conjugated Linoleic Acid (CLA) in Silkworm, *Bombyx mori*

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Abstract

Conjugated linoleic acid (CLA) exhibits potent anti-carcinogenic and other biological activities in several animal models. We report here that dietary CLA, chemically synthesized from corn oil, accumulates in silkworm, *Bombyx mori*, which is used as a therapeutic agent for diabetes in Korea and Japan. Mulberry leaves treated with 0.1 or 10% CLA in ethanol were supplied to silkworms from the end of the 3rd instar to the 3rd day of the 5th instar. Fresh mulberry leaves or leaves treated with 10% corn oil in ethanol were fed as a check treatment. The amount of total lipids in the larval body ranged from 17.4 to 19.1 mg/g of body tissue, which was not significantly affected by the source of the diets. No CLA was found in the control silkworms. But the level of CLA significantly increased to 83.5 mg/g of fat, when fed with mulberry leaves treated with 10% CLA. Only trace amounts of CLA were detected in the larvae reared with check leaves and 0.1% CLA-treated leaves. Mulberry leaves treated with corn oil or CLA were not palatable to the larvae, resulting in a reduction of larval weight. These results suggest that silkworms containing CLA in body lipids could be produced by dietary CLA.

Key words: conjugated linoleic acid (CLA) silkworm, *Bombyx mori*, accumulation, diabetes

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a group of positional and geometric (*cis*-9,*trans*-11-; *trans*-9,*cis*-11-; *trans*-9,*trans*-11-; *trans*-10,*trans*-12-; *trans*-10,*cis*-12-) configurations of octadecadienoic acid (C18:2) with a conjugated double bond system (1,2). Conjugated linoleic acid found in a variety of foods related to ruminant animals, has been reported to have anti-carcinogenic, hypocholesterolemic, and anti-artherogenic effects on several animal models (1,3-5). Liu and Belury (6) recently reported that CLA reduces tomorogenesis by depressing prostaglandin E_2 synthesis. Other significant biological activities, such as immune stimulation (7) and body fat reduction (8), were evident. Dietary CLA, composed mainly of *c*9-*t*11 CLA and *t*10-*c*12 CLA, is incorporated in phospholipids and neutral fat in the tissues of experimental animals (9), poultry (10), pork (11) and fish (12), and egg yolk (10,13). Recently, CLA accumulation in arthropods was reported in the bodies of house fly pupae and adults (14). However, no reports on the accumulation of dietary CLA in the body fat of silkworms were available in the literature.

It is well understood that the silkworm or its solvent extract has an effect of lowering blood glucose levels by inhibiting the intestinal glycosidase activity (15-17). Hence, silkworm powder is widely used in Korea for the treatment of sugar diabetes. The powder is also available in Japanese markets as tablets with the commercial name of "Bosulin" (Bombyx+ Insulin). If dietary CLA can be incorporated in silkworm tissue, the insect would be of considerable value as a therapeutic agent for cancers and sugar diabetes, as well as other effective biological functions.

We report here that CLA, containing *c*9-*t*11 and *t*10- *c*12 CLA, supplemented to mulberry leaves is substantially accumulated in silkworm body tissue.

MATERIALS AND METHODS

Preparation of CLA

The CLA was chemically synthesized from 95% linoleic acid (Dusan Lipid Co., Seoul, Korea) by the alkaline isomerization method described by Kim et al. (18). The CLA consisted of 47% *c*9-*t*11 CLA and 49% *t*10-*c*12 CLA isomers when analyzed by GC described below

Rearing of the insects

The silkworm (variety: tesungjam) were reared by conventional methods except for CLA treatment in the rearing room of the Kyoungnam Agricultural Research and Extension Center (Chinju, Korea). Silkworms were reared with fresh mulberry leaves until reaching the beginning of the 3rd instar. The larvae were fed with mulberry leaves treated with CLA solution from the end of the 3rd instar. The CLA was diluted in ethanol (>99.9%) at concentrations of 0.1 and 10.0% (v/v). The solution was sprayed on both sides of the mulberry leaves with a hand sprayer. The amount of treated CLA in

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the 0.1 or 10% solution was equivalent to 0.12 mg or 12.21 mg/g mulberry leaf, respectively. The leaves treated with CLA solution were kept at open door for 30~40 min to evaporate the ethanol and then stored in a basement until supplied to the larvae. Fresh mulberry leaves or leaves treated with corn oil (10% in ethanol) were supplied as controls. Each treatment was triplicated and 300 individuals were maintained per replicate. When the larvae reached the 3rd day of the 5th instar, they were weighed to check larval development, and then frozen in a home refrigerator until contents of fatty acids, including CLA, were determined.

Total lipid extraction and fatty acid analysis

Total lipid extraction

Whole bodies of silkworm larvae (5 to 6 larvae per replication) were weighed into a centrifuge test tube (40 mL volume) containing 10 mL chloroform:methanol (2:1, v/v) and 0.5 mg heptadecanoic acid (internal standard). Total lipids were extracted according to Folch et al. (19).

Preparation of fatty acid methyl esters

Three mL of 0.05 N H_2SO_4 in absolute methanol were added to a Teflon-lined screw-cap test tube (10 mL) containing 10 mg of the total lipid sample. The test tube was filled with nitrogen, then tightly capped and heated in a boiling water bath for 5 min. The hexane extract containing fatty acid methyl esters was dried over sodium sulfate anhydrous, filtered with a filter paper, and the hexane was evaporated.

GC analysis of fatty acid methyl esters

The GC employed for analysis of the fatty acid methyl esters was a Hewlett Packard 5890 equipped with Supelcowax-10 capillary column (60 m×0.32 mm, i.d., 25 μm film thickness, Supelco, PA, USA). The oven temperature was increased from 180°C to 220°C at a rate of 3°C/min. Temperatures of both injector and detector were 250°C. The carrier gas was N_2 (3 mL/min). Conjugated linoleic acid and fatty acid methyl esters of samples were identified by comparison of the standards and quantified by internal standard method using a heptadecanoic acid.

Statistical analysis

Data were analyzed using the Student-Newman-Keul's test (20) to compare individual treatment.

RESULTS AND DISCUSSION

The amount of total lipid in the silkworms fed with mulberry leaves treated with 0.1 or 10% CLA solution was not significantly different from that fed with fresh or corn oil-sprayed leaves. It ranged from 17.4 to 19.1 mg/g of larval body (Fig. 1). The content of CLA in the larvae fed with mulberry leaves sprayed with 10% CLA solution was significantly higher than that in control larvae, whereas only trace amounts of CLA were detected in the larvae fed with mulberry leaves sprayed with a 0.1% CLA solution (Fig. 2). The concentration of CLA isomers in the silkworms for *c*9-*t*11 and *t*10-*c*12 CLA was

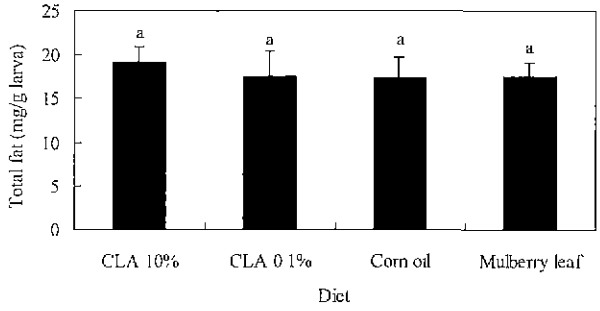

Fig. 1. Total lipid contents in silkworm fed with mulberry leaves treated with CLA. Mulberry leaves treated with or without corn oil (10% concentration) was supplied to the larvae as a check treatment. Means with the same letters on the bars are not significantly different by Student-Newman-Keul's test ($p<0.05$).

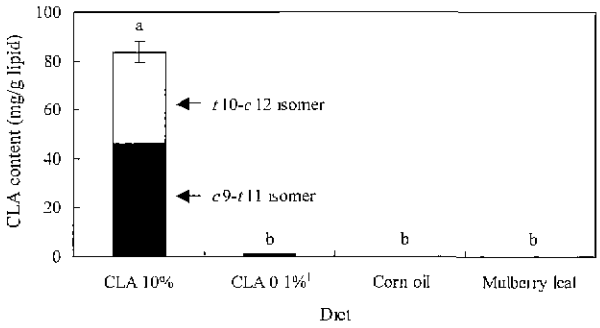

Fig. 2. Contents of total CLA in silkworm fed with mulberry leaves treated with CLA. The bar with an asterisk (*) represent only trace amount of CLA detected in silkworm fed with 0.1% CLA-treated leaves. Mulberry leaves treated with or without corn oil (10% concentration) was supplied to the larvae as a check treatment. Means with the same letters on the bars are not significantly different by Student-Newman-Keul's test ($p<0.05$).

46.3 and 37.2 mg/g of body fat, respectively. The amount of CLA accumulated in the silkworm larvae (83.5 mg/g of fat) (Fig. 2) was similar to that in egg yolk (10) from hens fed with dietary CLA, but much higher than that found in dairy products or cooked beef (3.1~8.5 mg/g of fat) (21,22). Synthetic dietary CLA isomers are incorporated in phospholipid and neutral fat fractions of fish, chicken and tissues of experimental mice (3,10,23). In these animal systems, a predominant CLA isomer incorporated in the phospholipid fraction is *c*9-*t*11 CLA, whereas neutral fat fractions contain all possible CLA isomers, including both *c*9-*t*11 CLA and *t*10-*c*12 CLA isomers. Our previous study showed that these CLA isomers accumulated in the bodies of house fly pupae and adults with no adverse biological effects (14).

CLA is naturally produced in ruminant animals such as cattle, sheep, and goats (21). The ruminant microorganism, *Butyrivibrio fibrisolvens*, is responsible for the synthesis of the *c*9-*t*11 CLA isomer as an intermediate in the biohydrogenation of linoleic acid to vaccenic acid (24,25). *Lactobacillus reuteri* has been found to synthesize *c*9-*t*11 CLA in human guts (26). No

article has reported insect species containing these bacteria in its intestines. The oleic, palmitic, stearic, linoleic and α-linoleic acids are the most abundant fatty acids in the Mediterranean arctiid moth *Cymbalophora pudica* (27). The house cricket and the American cockroach are able to produce linoleic acid, *de novo* (28). These insects possess a Δ^{12}-desaturase in their tissues, which is responsible for the conversion of oleic acid to linoleic acid. Borgeson and Blomquist (29) provided evidence that the Δ^{12}-desaturase activity in the two insect species is of insect origin and not due to endosymbiotic bacteria.

Mulberry leaves contain a great amount of palmitic, linoleic and linolenic acids which are essential to the growth of silkworms. The silkworm should uptake linoleic and linolenic acids from food, because they can not synthesize, *in vivo*, these two fatty acids (30). Even though some insect species can produce linoleic acid, there is no evidence that these species synthesize CLA from linoleic acid. Therefore, the CLA determined in these silkworms originated from the CLA sprayed on the mulberry leaves. This fact is established by the results which show that no CLA was detected in the silkworms fed with fresh mulberry leaves (Fig. 2). Even though we did not measure the CLA content incorporated in tissues and food stuffs in the alimentary canals of the silkworm separately, it is possibile that the dietary CLA was incorporated in the lipid fractions of silkworm tissues, as other fatty acids which are accumulated as triglyceride and phospholipid forms. Based on ongoing experiments a great amount of dietary CLA was distributed among silkworm body tissues, hemolymph and food stuff in the intestines. More detailed results will be published elsewhere.

Only small amounts of CLA were detected from the larvae fed with leaves sprayed with 0.1% CLA (Fig. 2). This might be, in part, due to metabolism of the CLA as an energy source of the insect, since excess amounts of CLA are metabolized via β-oxidation in animal tissues (31). Alternatively, it could be due to the detection limitation of samples containing other major fatty acids, as shown in Table 1. The content of fatty acids was not significantly different between treatments, except for that of palmitic acid (C16:0) which significantly decreased in the larvae fed with CLA-sprayed mulberry leaves (Table 1). The reason why the content of palmitic acid was decreased in the larvae can not be explained from this study.

Alcoholic CLA solution sprayed on the mulberry leaves significantly affected larval weight (Fig. 3). CLA-treated leaves were prepared daily and kept in a basement to maintain the

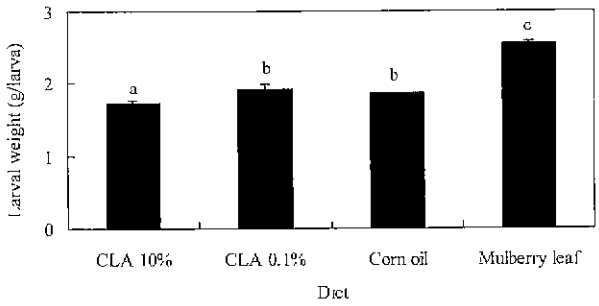

Fig. 3. Weight of silkworm larva fed with mulberry leaves treated with CLA. Mulberry leaves treated with or without corn oil (10% concentration) was supplied to the larvae as a check treatment. Means with the same letters on the bars are not significantly different by Student-Newman-Keul's test ($p<0.05$).

freshness; however, necrosis was shown in a certain portion of the leaves. It could be one of the reasons why there was a decrease in food intake, which led to a reduction in body weight. Our preliminary data showed that food intake reduced 35.3% in the silkworms fed with 10% CLA-treated leaves, compared with the insects fed with fresh leaves.

In conclusion, dietary CLA accumulated in silkworm body tissue. The body weight of the silkworms fed with leaves treated with CLA or corn oil was reduced because of food intake reduction. Further research is needed to develop supply methods of CLA to silkworms, if we want to produce, so called, 'CLA-silkworms' which might be used as a therapeutic agent for diabetes and cancers, with other biological effects.

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Table 1. Composition of fatty acids other than CLA in silkworm fed with various treatment of mulberry leaves (mg/g lipid)

Diets	Myristic acid	Palmitic acid	Stearic acid	Oleic acid	Linoleic acid	γ-linolenic acid	Linolenic acid
Mulberry leaf	3.3±1.4a	179.5± 9.0a	195.8± 1.6a	229.6±14.9b	101.3± 3.0a	0.6±0.2a	289.9± 9.5a
Corn oil (10%)	3.2±0.8a	172.2±20.3a	186.0±35.5a	282.1±19.8a	134.7±87.5a	0.9±0.5a	220.1±24.3b
CLA 0.1%	2.4±0.8a	140.2± 6.4b	232.1± 4.5a	193.7± 1.6c	131.6± 1.7a	0.9±0.2a	299.2± 4.5a
CLA 10%	2.4±1.0a	148.1±10.5b	205.0± 1.4a	228.5± 2.5b	94.6± 5.8a	1.4±0.5a	236.6± 4.3b

Means followed by the same letter in the same column are not significantly different by Student-Newman-Keul's test ($p<0.05$).

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