Lipid Profiles and Oxidative Stability of Silkworm Pupal Oil

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Abstract: The oil content of the silkworm, Bombyx mori, in the pupal stage was 4.8% for the male and 9.0% for the female (wet basis), respectively. Total lipid (TL) extracted from silkworm pupae mainly consisted of triacylglycerol (TG), phosphatidylethanolamine, and phosphatidylcholine. TL and TG contained approximately 40% α-linolenic acid as the predominant fatty acid. 20:3n-3 was also present in the pupa, but the quantities were very small. Total tocopherol in TL was 125.2 µg/g lipid for the male and 224.1 µg/g lipid for the female, respectively. The oxidative stability of TL was confirmed by measuring the decrease in oxygen in the headspace. The oxidative stability of TL from silkworm pupae was very high and no difference between sexes was appeared. It has been suggested that a synergistic effect between phospholipids and tocopherol in silkworm pupa plays an important role in protecting the lipids against oxidation. Furthermore, the silkworm pupa contained carotenoids such as lutein and neoxanthin. These carotenoids may act as antioxidants in TL. The silkworm pupa would be a good source of the functional fatty acid, α-linolenic acid, and of the functional pigments, lutein and neoxanthin.

Key words: Bombyx mori, silkworm pupa, oxidative stability, α-linolenic acid, antioxidant

1 Introduction

Sericulture-related compounds, other than silk, can be resources for many products such as functional foods, cosmetics, and pharmaceuticals. Feces of the silkworm and root bark of the mulberry tree have been used as Chinese traditional medicines. Silkworm powder and mulberry leaves contain alkaloids such as 1-deoxynojirimycin, and these alkaloids were reported to be inhibitors of glucosidase (1, 2). Fagomine is suggested to potentiate glucose-induced insulin secretion in isolated rat pancreatic islets (3). 2-O-α-D-galacto-pyranosyl-deoxynojirimycin had a strongly antihyperglycemic effect in diabetic mice (4). In fact, mulberry leaf powder decreased the human blood glucose level (5). Silk protein, sericin, was shown to suppress lipid oxidation of rat brain homogenate and to inhibit tyrosinase activity (6). Silkworm and mulberry trees have therefore provided not only silk thread but also various useful compounds for us. On the other hand, after the processing of silk reeling, the silkworm pupa becomes an industrial waste. Therefore, new uses of silkworm pupa have not been greatly developed except for aquacultural diets or local foods in Japan and effective use of silkworm pupa has been expected.

Silkworms ingest mulberry leaves as diet and accumulate lipids in the body (7) for various purposes such as energy resources, nutrimental resources for egg formation (8) and cuticular lipids. Mulberry leaves contain α-linolenic acid as the main fatty acid, so that the main
fatty acid in each stage of the silkworm is also \( \alpha \)-linolenic acid (7). \( \alpha \)-Linolenic acid has been reported to have various beneficial biological actions (9-19) and silkworm pupae are a possible oil source containing a large amount of this functional polyunsaturated fatty acid. In the present study, we evaluated the lipid profiles and the fatty acid composition of the oil extracted from the silkworm pupa.

On the other hand, \( \alpha \)-linolenic acid is susceptible to autoxidation leading to the formation of harmful substances (20), so that silkworm oil in pupa may contain a lot of antioxidants such as \( \alpha \)-tocopherol and other kinds of lipid-soluble antioxidants.

Carotenoids are present in the chloroplast of higher plants. The presence of the carotenoid-binding protein is known in the silkworm (21), so that carotenoids are also expected to be accumulated in the silkworm body. Carotenoids have attracted much attention for their beneficial effect on human health. Carotenoids are well known as dietary compounds with a free radical scavenging effect (22), singlet oxygen quenching effect (23) and activity preventing various types of cancer in culture cells (24-29), laboratory animals (30-32), and epidemiological studies (33, 34). In the present study, we also evaluated the carotenoid profile in the silkworm pupa as well as the \( \alpha \)-tocopherol. The pupal oil may include several functional lipid resources. The present study may indicate effective uses for hitherto unused resources.

2 Experimental

2.1 Materials
Soybean oil and linseed oil were purchased from Nacalai Tesque (Kyoto, Japan). Tocopherols were purchased from Eisai Co., Ltd. (Tokyo, Japan). Silkworm raw pupae used in following experiments were provided in the sericultural season in 2001 by the Institute of Sericulture, Dainippon Silk Foundation (Ibaraki, Japan).

2.2 Extraction of Lipids from the Silkworm Pupae and Separation of the Lipids into Some Lipid Classes
Total lipids (27 g for the male and 51 g for the female) were extracted from silkworm pupae (570 g wet basis) with chloroform/methanol/water according to the method of Bligh and Dyer (35). A quantity of the lipids was separated into some lipid classes by silicic acid column chromatography eluting with chloroform, acetone, and methanol, respectively. Column chromatographic separation was monitored by thin layer chromatography (TLC) according to authentic standards. TLC was carried out on 0.25 mm silica gel plates (Merck, Darmstadt, Germany), developed with chloroform/methanol/water (65:25:4, v/v) for the methanol fraction and with diethyl ether/n-hexane (30:70, v/v) for the chloroform fraction, respectively. The chloroform fraction was further fractionated on a silicic acid column, eluting with diethyl ether/n-hexane. Purified triacylglycerol (TG) was eluted with ether/n-hexane (10:90, v/v) and the TG gave only a single spot on TLC. Purified phosphatidylethanolamine (PE) and phosphatidylcholine (PC) were obtained by TLC separation of fractions eluted with methanol. TLC was carried out developing with chloroform/methanol/water (65:25:4, v/v) for PE and chloroform/methanol/water (25:10:1, v/v) for PC, respectively.

2.3 Analysis of Fatty Acid Composition and Tocopherol Content in the Lipids from Silkworm Pupae
Fatty acid compositions of total lipids, TG, PE and PC were analyzed by capillary gas chromatography (GC) after conversion of fatty acyl groups in each lipid to their methyl esters by heating in a sealed tube at 90 \( \sim \) 100°C for 1 h with 7% boron trifluoride (BF\(_3\)) in methanol under nitrogen. The GC analysis was performed with a Shimadzu GC-14 B gas chromatograph (Shimadzu Seisakusho Co., Kyoto, Japan) equipped with a fused-silica capillary column (Omegawax 320; 30 m \times 0.32 mm i.d.: Supelco, Bellefonte, PA, USA). The injection port and flame ionization detector were operated at 250°C and 260°C, respectively, the column temperature being held at 200°C. Tocopherol content in total lipids was determined by HPLC with a fluorescence spectrophotometer as described below (36). The tocopherols contained in the lipids were identified from their retention times and were quantified with calibration curves for the corresponding tocopherol standards. HPLC analysis was carried out in an L-7100 liquid chromatograph equipped with an L-7485 fluorescence spectrophotometer (Hitachi Seisakusho Co., Tokyo, Japan) at an excitation of 298 nm with an emission of 325 nm on a normal-phase column (250 \( \times \) 4.6 mm i.d. stainless steal; 3 \( \mu \)m particle size) packed with silica gel (Develosil-30-3; Nomura Chemical Co., Seto, Japan).
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The analysis was done isocratically with a mixture of n-hexane/2-propanol (99.2/0.8, v/v) as a mobile phase at a constant flow rate of 1 mL/min.

2.4 Oxidation of Lipids

Oxygen consumption by lipid oxidation was determined by GC (Shimadzu GC-14B) equipped with a thermal conductivity detector and a stainless steel column (3 m × 3.0 mm i.d.) packed with molecular Sieve 5A (GL Science Co. Ltd., Tokyo, Japan). Each lipid sample (20 mg) was put into a 1 mL aluminium-sealed vial with a butyl-gum septum and then incubated at 50 °C in the dark. The level of oxygen in the headspace gas in the vial was calculated from the peak ratio of oxygen to nitrogen.

2.5 Extraction of Carotenoid from Silkworm Pupae and Mulberry Leaves (Morus alba L.)

Acetone was added to the silkworm pupae (250 g wet basis) or small chips of mulberry leaves (250 g wet basis) and the mixture was homogenized. The acetone was evaporated with a rotary evaporator and the extracts were dissolved into ethyl acetate. The ethyl acetate was further evaporated with a rotary evaporator, and the carotenoid extracts were obtained.

2.6 HPLC Analyses of Major Carotenoids in the Extracts

The carotenoid extracts were analyzed by HPLC in a model 1100 HPLC system with a photodiode array detector (Hewlett-Packard, Palo Alto, CA) on an ODS-80Ts column (250 × 4.6 mm; Tosoh, Tokyo, Japan). The solvent system consisted of acetonitrile/methanol/water (65:15:20, v/v) containing 0.1% ammonium acetate (solvent A) and methanol/ethyl acetate (70:30, v/v) containing 0.1% ammonium acetate (solvent B). After isocratic elution with solvent A (100%) for 30 min, a linear gradient from solvent A (100%) to solvent B was applied for 10 min at a flow rate of 1 mL/min, followed by isocratic elution with solvent B (100%) for an additional 15 min. Peaks corresponding to carotenoids were monitored at 440 nm. Each carotenoid was quantified from its peak area by means of a calibration curve for the corresponding standards.

3 Results

3.1 Lipids Profiles from Silkworm Pupae

Total lipid (TL) content of silkworm pupae was 4.8%
for males and 9.0% for females (wet basis), respectively, showing higher TL content in females than in males. As shown in Table 1, most of TL was eluted with chloroform by silicic acid chromatography. The chloroform fraction mainly consisted of TG and contained small amounts of cholesterol (Fig. 1 A). On the other hand, the methanol fraction constituted about 10% of the total lipid (Table 1) and mainly consisted of PE and PC (Fig. 1 B). The male and the female gave similar lipid profiles, whereas the content of methanol fraction in the female was slightly higher than that in the male.

3.2 Fatty Acid Compositions of TL, TG, PE and PC

Fatty acid compositions of TL, TG, PE and PC from silkworm pupae are shown in Table 2. Little difference was found in the fatty acid compositions of TL, TG, PE and PC from male and female silkworm pupae. The predominant fatty acid of these lipids was 18:3n-3 (33.7%-42.7%). Therefore, it is clear that the most important characteristic of the lipids from silkworm pupae was the high levels of 18:3n-3, whereas the level was lower than that in a commercial linseed oil (Table 3).

3.3 Tocopherol in Lipids

As shown in Table 4, the total tocopherol content of the pupa TL (female) was approximately twice that of the pupa TL (male). In the pupa TL, \( \alpha \)-tocopherol was the most predominant species, followed by \( \gamma \), \( \delta \)-, \( \beta \)-tocopherol. After separation by silicic acid column chromatography, total tocopherol content in pupa TG decreased about one third to half the content in pupa TL. In particular, most of the \( \delta \)-tocopherol was removed by the chromatography. Furthermore, total tocopherol content in pupa TG was lower than in commercial soybean oil and linseed oil containing \( \gamma \)-tocopherol as the predominant tocopherol.

3.4 Oxidative Stability of Lipids

The oxidative stability of four kinds of lipids from silkworm pupae and two commercial oils were com-

| Table 2 | Fatty Acid Compositions of Total Lipid (TL), TG (Triacylglycerol), Phosphatidylethanolamine (PE) and Phosphatidylcholine (PC) from Silkworm Pupae. |
|---|---|---|---|---|---|---|
| Fatty acid (wt%) | Male | | | Female | | |
| | TL | TG | PE | PC | TL | TG | PE | PC |
| 16 : 0 | 24.9 | 23.1 | 8.5 | 12.2 | 19.5 | 21.4 | 9.3 | 11.0 |
| 18 : 0 | 5.4 | 4.1 | 8.9 | 11.1 | 6.3 | 4.7 | 13.7 | 13.6 |
| 16 : 1n-7 | 0.8 | 0.5 | 0.2 | 0.4 | 0.6 | 0.5 | 0.1 | 0.2 |
| 18 : 1n-9 | 24.3 | 20.6 | 20.4 | 14.8 | 22.6 | 21.9 | 24.0 | 17.3 |
| 18 : 2n-6 | 6.3 | 6.4 | 15.1 | 18.4 | 7.7 | 6.9 | 13.2 | 16.5 |
| 18 : 3n-3 | 36.0 | 42.7 | 41.3 | 38.5 | 40.7 | 41.8 | 33.7 | 36.3 |
| 20 : 3n-3 | 0.2 | 0.1 | 0.1 | 0.2 | 0.3 | 0.1 | 0.1 | 0.2 |

| Table 3 | Fatty Acid Compositions of Commercial Soybean Oil and Linseed Oil. |
|---|---|---|
| Fatty acid (wt%) | Soybean | Linseed |
| 16 : 0 | 10.6 | 5.5 |
| 18 : 0 | 3.5 | 3.1 |
| 16 : 1n-7 | 0.1 | 0.1 |
| 18 : 1n-9 | 20.5 | 17.8 |
| 18 : 2n-6 | 54.4 | 14.2 |
| 18 : 3n-3 | 6.5 | 56.2 |
| 20 : 3n-3 | — | 0.1 |

| Table 4 | Tocopherol in Lipids from Silkworm Pupae, Commercial Soybean Oil and Linseed Oil. |
|---|---|---|---|---|---|---|---|
| Lipids | \( \alpha \) | \( \beta \) | \( \gamma \) | \( \delta \) | Total |
| Pupa TL (male) | 73.3 | 8.5 | 23.1 | 20.3 | 125.2 |
| Pupa TL (female) | 131.0 | 15.0 | 42.1 | 36.0 | 224.1 |
| Pupa TG (male) | 33.2 | 11.9 | 23.6 | 1.0 | 69.7 |
| Pupa TG (female) | 40.6 | 13.2 | 20.1 | 1.0 | 75.0 |
| Soybean Oil | 53.3 | 7.3 | 196.0 | 8.7 | 265.3 |
| Linseed Oil | 4.0 | — | 235.1 | 5.1 | 244.2 |

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pared in the bulk phase by measuring the decrease in oxygen in the headspace. As shown in Fig. 2, both pupa TLs were the most oxidatively stable among lipids tested in the present study. Little decrease in oxygen in the headspace of these TLs was observed during incubation for 771 h. No difference was apparent between sexes of silkworm pupae in the oxidative stability of TL. On the other hand, the female was more stable than the male in the oxidation of pupa TG. After 82h oxidation, the oxygen concentration in the headspace was 95% for the female TG and 39% for the male TG. The oxidative stability of the female TG was comparable to that of linseed oil, but lower than that of soybean oil.

3.5 Carotenoid Profiles in Silkworm Pupae

Three major peaks for the extracts from mulberry leaves and four major peaks for the extracts from silkworm pupae were detected at 29-45 min (Fig. 3 A and B). The peaks at 34.2 min, 40.7 min and 44.1 min were assigned as neoxanthin, violaxanthin, and lutein, respectively, based upon their UV-visible (VIS) spectra and the retention times of standard peaks as shown in Fig. 3 (C). The $\lambda_{\text{max}}$ values in their UV-VIS absorption spectra were 414, 438, and 466 nm for neoxanthin, 418, 442, and 470 nm for violaxanthin, and 423, 446, and 474 nm for lutein, respectively (Fig. 4). The amounts of neoxanthin, violaxanthin, and lutein were 0.22 mg, 0.07 mg, and 1.28 mg for the silkworm pupae at 250 g (wet basis) and 6.78 mg, 2.41 mg, and 11.33 mg for the mulberry leaves at 250 g (wet basis), respectively.

The peak indicated by an arrow in Fig. 3 (A) was eluted at 29.3 min and the $\lambda_{\text{max}}$ values in the UV-VIS absorption spectra were 418, 442 and 470 nm (Fig. 5).
Sericulture-related products from silk, silkworm and mulberry leaves can be regarded as good sources of nutriments and pharmaceuticals. Expression of useful protein and hormone in silkworms has also been attempted in several studies (37, 38) and further studies on new uses of sericulture-related compounds have been expected. In the present study, we have tried to find some health-related functional compounds in silkworm pupa. The TL content of female pupae was higher than that of the male. The development of eggs may require more lipids. Silkworm oil in pupa mainly consists of triacylglycerol and phospholipids such as PE and PC. The main fatty acid in these lipids was \( \alpha \)-linolenic acid. Several vegetable oils such as citrus seed oil, taramira seed oil and Canadian weed seed oil other than linseed oil are known as the oils containing a high level of \( \alpha \)-linolenic acid (39-41). But, animal oils containing a high level of \( \alpha \)-linolenic acid are little known, whereas horse milk fat contains 16% of \( \alpha \)-linolenic acid (42). Of particular interest is the high level of \( \alpha \)-linolenic acid in pupal oil as an animal one. \( \alpha \)-Linolenic acid has been found to inhibit the growth of several cancer cell lines, such as colon (9), larynx (10), prostate (11), pancreatic (12) and leukemia (13). Furthermore, it has been suggested that \( \alpha \)-linolenic acid suppresses as the development of allergies (15,16), obesity (17) and neuronal death (19). Pupal oil would therefore be a good source of the functional fatty acid, \( \alpha \)-linolenic acid.

On the other hand, due to its high degree of unsaturation, \( \alpha \)-linolenic acid is known to be oxidatively less stable and the oxidation of \( \alpha \)-linolenic acid is one of the major problems in the use of oils containing a high level of \( \alpha \)-linolenic acid in food and nutraceuticals. Nevertheless, TL from silkworm pupae were oxidative-ly much more stable than soybean oil, although the fatty

4 Discussion

Sericulture-related products from silk, silkworm and mulberry leaves can be regarded as good sources of nutriments and pharmaceuticals. Expression of useful protein and hormone in silkworms has also been attempted in several studies (37, 38) and further studies on new uses of sericulture-related compounds have
acid composition of these oils had suggested the lower stability of silkworm pupa TL. Antioxidants, especially tocopherol content, are the most important factor affecting the oxidative stability of oils. The main tocopherol species of TL from silkworm pupae was \( \alpha \)-tocopherol and the total tocopherol content of both pupa TLs was lower than that of commercial oils, soybean oil and linseed oil, but among the various oils tested, pupa TLs were the most oxidatively stable and little oxygen consumption was observed during the incubation. It has been reported that each tocopherol species has different antioxidant activity and \( \delta \)-tocopherol is known to be the most active in bulk oil, followed by \( \gamma \)- and \( \alpha \)-tocopherol, respectively (43,44). The main tocopherol species of pupa TL was \( \alpha \)-tocopherol, whereas that of soybean oil or linseed oil was \( \gamma \)-tocopherol. These results suggest that the higher oxidative stability of pupa TL could not be explained by the content and species of tocopherol itself.

Phospholipids have been reported to have antioxidant activity (45-48) and to have synergistic effects together with tocopherol (49-52). High content of phospholipids in pupa TL would play an important role in the protection of the TL against oxidation. Furthermore, we found the presence of some kinds of carotenoids such as lutein and neoxanthin. Neoxanthin was reported to be 100-fold more active than \( b \)-carotene in its antioxidant activity on malondialdehyde formation from human red blood cells (53). Carotenoids may also be strongly correlated with the higher oxidative stability of pupa TL found in the present study.

On the other hand, the oxidative stability of male and female pupa TGs was lower than that of soybean oil. When the oxidative stabilities of both pupa TGs were compared, male pupa TG was oxidized more rapidly than female pupa TG. The average bisallylic number of male pupa TG (2.79) was slightly higher than that of female (2.76) and total tocopherol content of male pupa TG (69.7) was lower than that of female (75.0). These differences, mainly the lower tocopherol content, would be due to the lower stability of male than that of female in the oxidation of pupa TG.

Several reports have shown that these carotenoids have beneficial effects on cancer chemoprevention. Lutein was the most predominant carotenoid in silkworm pupa and mulberry leaves, followed by neoxanthin and violaxanthin. Lutein was reported to be absorbed and accumulated in human plasma and various tissues as one of the predominant carotenoids (54), and to induce differentiation in HL-60 cells (55) and apoptosis in SV-40 transformed mammary cells and MCF-7 human mammary carcinoma cells (56). Chang and Lin reported that neoxanthin strongly inhibited cell growth by suppressing DNA synthesis in C3H10T1/2 cells (53), and that neoxanthin inhibited 7,12-dimethylbenz [a] anthracene (DMBA)-induced carcinogenesis in the hamster buccal pouch (57). We recently found that neoxanthin and violaxanthin reduced the viability of human prostate cancer cells, PC-3, DU 145, and LNCaP (58). Furthermore, in the present study, we found that a carotenoid was present only in the pupa, but not present in mulberry leaves, whereas we have not identified the carotenoid. Because carotenoids cannot be synthesized in animals including insects, the unidentified carotenoid may be metabolized from other carotenoids such as neoxanthin or violaxanthin.

The identification and the biological effects of the unidentified carotenoid also deserve future studies.

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