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Comparison of nutrient compositions and pharmacological effects of steamed and freeze-dried mature silkworm powders generated by four silkworm varieties

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ABSTRACTS

The mulberry silkworm, *Bombyx mori*, has provided valuable fabrics and foods to humans for > 5000 years. We invented the protocol for processing mature silkworms, which contain various functional substances, into edible steamed and freeze-dried mature silkworm powders (SMSPs). However, previously reported technique could not create powders smaller than 0.1 mm and had problems with loss of product due to large SMSP particles sticking to hammer mill machines during the pulverizing process. To resolve these problems, we invented a new pulverization protocol that could create particle sizes smaller than 0.01 mm. Reduced particle sizes in SMSPs offered several advantages: increased nutrient contents in the nutritional aspect and extended life expectancy and enhanced locomotor activity in the pharmacological aspect. In addition, four SMSPs were enriched with flavonoids, poly-phenols, and vitamins that are known to act as oxidative stress inhibitors in cells and tissues. Nutrient and phytochemical composition analysis results suggested that this is why SMSPs extended healthspans and inhibited onset of Parkinson's disease. Although most nutrient and phytochemical compositions among the four SMSPs were comparable, certain nutrients and chemicals were significantly higher in certain SMSPs. Therefore, further research using these four SMSPs will identify the specific health-promoting effects of each SMSP, resulting in the further development of sericulture and entomology.

Introduction

The mulberry silkworm, *Bombyx mori*, has been supplying various byproducts to humans since it was domesticated 5000 years ago (Cherry, 1989). The cocoons of silkworms have been used for manufacturing high-quality natural fabrics, and its pupae have been used as a source of proteins and oils. In addition, it has long been known that the silkworms and the mulberry leaves have been used as natural medicines in East Asian countries because of their various health-promoting effects (Nguyen et al., 2016; Ryu et al., 1997). The silkworm and mulberry leaves have been made into powders after freeze-drying to facilitate human consumption (Ji et al., 2015; Ryu et al., 1997).

The currently produced silkworm varieties are F1 hybrids with excellent traits that are created by crossing two out of the 340 pure breeds (PBs) maintained by the National Academy of Agricultural Sciences (NAAS), Korea. The White jade (WJ) variety, which is also known as Baegokjam, is the F1 hybrid of Jam 123 and Jam 124 PBs and produces relatively large white cocoons (Lee et al., 1984). The Golden Silk (GS) variety, which spins yellow cocoons, is the F1 hybrid of Jam 311 and Jam 312 PBs (Kang et al., 2007). Pistachio silk (PS) variety, which is also known as Yeonnokjam is the F1 hybrid between Jam 315 and Jam 316 PBs and produces light-green cocoons (Kang et al., 2011; Kim et al., 2017). The Red Silk (RS) variety is the F1 hybrid between BPred and Jam 157 PBs (Ji et al., 2016a).

Recently, we examined the differences in nutritional composition according to developmental stages and production methods using the WJ variety. Compared with freeze-dried 3rd day of 5th instar silkworm powder or freeze-dried mature silkworm powder, steamed and freeze-dried mature silkworm powder (SMSP) has a high content of crude protein and important amino acids (Ji et al., 2016b). In addition, the

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nutrition composition analysis results of GS-, PS-, and RS-SMSPs revealed that certain amino acids, minerals, and fatty acid content varied among them, although most nutritional values were comparable to one another (Ji et al., 2016a).

Recently, several health improvement effects of SMSPs have been reported. For example, WJ-SMSP extended healthspan, increased resistance to Parkinson's disease (Nguyen et al., 2016), and had a protective effect on diethylnitrosamine-induced hepatotoxicity in animal models (Cho et al., 2016). PS-SMSP reduced UV-induced melanization of skin in animal models (Kim et al., 2017). These reports suggest that SMSPs generated from different varieties with unique cocoon colors may have additional, though as yet unknown, health-improvement effects.

A recent study on the mechanisms underlying silkworm cocoon color variations has shown that differences in transporting carotenoids derived from mulberry leaves in midgut lumens into the middle silk gland caused different cocoon colors (Tsuchida and Sakudoh, 2015). Thus, it is possible that cocoons with varied colors may accumulate different kinds of phytochemicals derived from the mulberry leaves. However, there has not yet been a report of differences in accumulated phytochemicals in the silk glands of mature silkworms.

In this study, we developed improved SMSP production protocols and performed analyses of nutrition components, phytochemicals, and vitamins for four SMSPs produced by a new protocol. Our results suggested that a new pulverization method can produce much smaller-sized SMSPs with a higher content of nutrients, phytochemicals, and vitamins. In addition, there were differences in the contents of phytochemicals and vitamins among four SMSPs. Those differences may be responsible for unique health-improvement effects among SMSPs.

Material and methods

Silkworm rearing protocol

The previously published four varieties of mulberry silkworm, *Bombyx mori*—White Jade (WJ, also known as Baekokjam), Golden Silk (GS), Pistachio silk (PS, also known as Yeonnokjam), and Red Silk (RS)—were raised with mulberry leaves at the NAAS campus at Wanju-gun, Jellabuk-do, Korea, and harvested as previously published by Ji et al. (2017).

Designing a new steamed and freeze-dried mature silkworm larval powder (SMSP) processing protocol

The mature silkworm larvae of four varieties were subjected to steaming for 130 min using an electric pressure-free cooking machine (Kum Seong Ltd., Boocheon, Korea) and freeze-drying with a freeze-drier (FDT-8612, Operon Ltd. Kimpo, Korea) at − 50 °C for 24 h, as previously published by Ji et al. (2015). The pulverization protocol was newly designed in this study. The steamed and freeze-dried mature silkworm (SMS) was cut to a size of 1–5 mm using a multipurpose mill (DSMP-370, DukSan Co., Ltd., Siheung, Korea) and then pulverized into particles having a size of 0.01 mm or less using a natural-stone roller mill (Duksan Co.) (Supplementary Fig. 1).

Proximate analysis for determining crude nutrients in four SMSPs

The proximate analyses for determining crude nutrients in four SMSPs were performed as previously published (Ji et al., 2016a, 2016b). Four SMSPs were completely dried at 105 °C under atmospheric pressure to determine the amount of H_2O. The amounts of crude proteins in four SMSPs were determined by semi-micro-Kjeldahl methods using a Kjeltec 2400AUT automatic protein analyzer (Kieltec, Poss Tector, Mulgrave, Australia). The contents of crude lipid in four SMSPs were analyzed by a Soxtec HT1043 extraction unit (Soxtec System, Poss Tectator) after being extracted by diethyl ether. The remains after diethyl ether extraction were digested with 1.25% H_2SO_4 and 1.25% NaOH to determine the content of crude fibers.

Amino acid composition analysis of four SMSPs

The protocols in the Korea food code used in a previous publication (Ji et al., 2016a, 2016b) were employed to quantify the content of amino acids. For quantifying the contents of Cysteine (CYS) and Methionine (MET), equal amounts of SMSPs were mixed with 20 ml of formic acid, left at 4 °C overnight to remove volatile compounds and then mixed with 6 N HCl for protein hydrolysis (quantification solution I). Quantification solution II used to quantify all amino acids except CYS, MET, and Tryptophan (TRP) was generated by blowing N_2 gas into the sample, followed by hydrolysis of proteins with 6 N HCl at 110.0 ± 1.0 °C for 22.0 ± 1.0 h. A rotary evaporator was used to remove HCl in quantification solutions I and II. Solutions neutralized by adding H_2O were concentrated by a rotary evaporator. The amino acid contents were measured by a Hitachi L-8900A automatic amino acid analyzer (Hitachi, Tokyo, Japan). To quantify TRP, samples mixed with 20 ml of 4.2 N NaOH were blown with N_2 gas and then hydrolyzed at 110.0 ± 1.0 °C for 22.0 ± 1.0 h. Samples were neutralized with 6 N HCl, and then 0.2 N sodium citrate solution was used to adjust the pH of samples to 4.25. The quantification of TRP was accomplished by a Hitachi L-8900A (Hitachi) automatic amino acid analyzer according to the manufacturer's protocol.

The quantification of minerals in four SMSPs

The protocol from the Association of Official Analytical Chemist (4) was used to determine the amounts of minerals in the samples, where pre-incinerated samples were completely incinerated at 600 °C for 2.0 h. After being cooled to RT, 0.5 g of sample was mixed with 10 ml of 50.0% HCl, incubated overnight and then filtered with No. six filter paper (GE Healthcare, Life Sciences, Chicago, IL, USA) with hot water. The PerkinElmer Optima 8300 (PerkinElmer Corporation, Norwalk, CT, USA), an inductively coupled plasma optical emission spectrometer, was used to quantify minerals by detecting the wavelength and intensities of specific emitted radiation rays for each mineral.

The quantification of mono- and di-saccharides in four SMSPs

To determine five mono- or di-saccharides, including glucose, fructose, sucrose, maltose, and lactose in four SMSPs, samples mixed with 15 ml of 50% ethanol (EtOH) were vortexed, sonicated for 20 min in an 80 °C water bath, cooled on ice for 3 min, shaken for 15 min with 2000 rpm, and then spun down for 10 min at 3000 rpm. Samples filtered by syringe filters with 0.2-μm pore size were analyzed by a Shiseido Nanospace SI-2 (Shiseido, Tokyo, Japan) refractive index detector using a Unison UK-Amino (5 × 2 mm, 3 μm, Imtakt Corp, Kyoto, Japan) as a guard cartridge and Unison UK-Amino (250 × 3 mm, 3 μm, Imtakt) as an analytical column. The mobile phase was 90% acetonitrile with a column temperature of 60 °C and a flow rate of 0.4 ml/min. No mono- or di-saccharides were detected in the four SMSPs (data not shown).

Identification and quantification of fatty acids in four SMSPs

The Folch method (Folch et al., 1957) was used to extract fatty acids from the four SMSPs. Briefly, 250 ml of reaction solution (chloroform:MeOH = 2:1) and 50.0 g of samples were mixed and homogenized to extract fatty acids. Extracted fatty acids were dehydrated by anhydrous Na_2SO_4 and concentrated at 50–55 °C. After adding 1 ml of tricosanoic acid and 1 ml of 0.5 N NaOH sequentially, the samples were boiled for 20 min at 100 °C. Cooled samples were mixed with 2 ml of BF3-MeOH, heated for 20 min and then cooled down to RT before adding 1 ml of heptane and 8 ml of NaCl. The supernatants were