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Nutrition composition differences among steamed and freeze-dried mature silkworm larval powders made from 3 *Bombyx mori* varieties weaving different colored cocoons

Sang-Deok Ji[1], Nam-Suk Kim[2], HaeYong Kweon[1], Bo Hye Choi[3,4], Kee-Young Kim[2*] and Young Ho Koh[3,4*]

[1]*Department of Agricultural Biology, National Institute of Agricultural Science, Rural Development Administration, Wanju-gun, Jeollabuk-do, Republic of Korea*
[2]*Research Policy Bureau, Rural Development Administration, Jeonju-si, Jeollabuk-do, Republic of Korea*
[3]*Department of Biomedical Gerontology, Graduate School of Hallym University, Chuncheon, Gangwon-do, Republic of Korea*
[4]*Ilsong Institute of Life Science, Hallym University, Anyang, Geyeonggi-do, Republic of Korea*

Abstract

The mulberry silkworm, *Bombyx mori* has been one of the most important domestic animals which have provided with silk fibers for weaving fabrics and a food for a protein and lipid source. In addition, various health improvement effects of diverse silkworm larval powders were reported. Recently we found that steamed and freeze-dried mature silkworm larval powder (SMSP) generated using white-jade (also known as Backokjam) silkworm variety extended healthspan and increased resistance to Parkinson's disease in animal models. Because the colors of cocoons in silkworm varieties were caused by altered signal transduction pathways transporting phytochemicals from intestinal lumens to silk glands, we performed the proximate, amino acid, mineral, carbohydrates, fatty acid, and cholesterol composition analyses of SMSPs of 3 silkworm varieties which were weaving light yellow, golden, and red cocoons. Although most of nutrient compositions among 3 SMSPs were similar, there were significant differences in certain amino acids, minerals, and fatty acid compositions. Red silk silkworm (RS)-SMSP had higher contents of crude proteins and total amino acids than other SMSPs. In addition, the ratio of n-3/n-6 unsaturated fatty acids were higher than the other SMSPs. In contrast Golden silk silkworm (GS)-SMSP had higher ratio of potassium/sodium than the other SMSPs. These nutrient analysis results suggested that 3 SMSPs might have common and unique health improvement effects. Thus, further studies in the functionalities of the 3 SMSPs will reveal unknown their health improvements effects.

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***Corresponding author.**
Kee-Young Kim, Ph.D.
Research Policy Bureau, Rural Development Administration, Jeonju-si, Jeollabuk-do, Republic of Korea
Tel: +82-63-238-0579 / FAX: +82-63-238-1777
E-mail: applekky@korea.kr
Young Ho Koh, Ph.D.
Department of Biomedical Gerontology, Graduate School of Hallym University, Chuncheon, Gangwon-do, Republic of Korea
Tel: +82-31-380-158 / FAX: +82-31-388-3427
E-mail: kohyh@hallym.ac.kr

Introduction

In addition to producing silk threads, *Bombyx mori* the mulberry silkworms have been used as producing functional foods with various health improvement effects (Ji *et al.*, 2012; Kim *et al.*, 2005; Nguyen *et al.*, 2016; Oh *et al.*, 2012; Ryu *et al.*, 2002; Ryu *et al.*, 2013). The most well-known health improvement effects of *Bombyx mori* silkworms was controlling blood glucose levels in humans and animal models when freeze-dried 3rd day of 5th instar larval powders (FDSP) were regularly taking (Ryu *et al.*, 2013). Recently, we also reported that steamed and freeze-dried mature silkworm larval powder (SMSP) had another health improvement effects such as extended healthspan and increase resistance to Parkinson's disease (PD) in *Drosophila* models (Nguyen *et al.*, 2016). In addition, we also found that amino acid compositions and mineral contents of FDSP and SMSP were different, since FDSP and SMSP were generated from different developmental stages of silk worm larvae (Ji *et al.*, 2016). The amount of crude proteins and amino acid composition differences between them were caused by enriched silk fibers in enlarged silk glands in mature silkworm larvae (Ji *et al.*, 2016). All tested silkworm larval powders contained high amounts of Ω-3 (n-3) unsaturated fatty acids, which are one of the most popular health improvement supplements (Walz *et al.*, 2016).

National Institute of Agricultural Science (NIAS) is securing total 340 silkworm pure breeds that have various phenotypes such as colors and sizes of cocoons, female's fecundity, etc. By hybridizing those pure breeds, NIAS developed several silkworm varieties producing light green, yellow, or red cocoons (Kang *et al.*, 2007; Ryu *et al.*, 2013). The Golden silk (GS) silkworm variety producing yellow cocoons was F1 hybrid between Jam311, a Japanese pure breed and Jam312, a Chinese pure breed which was spinning yellow silk fibers (Kang *et al.*, 2007). Yeonnokjam (YNJ) silkworm variety producing light green colored silk fibers was F1 hybrid between Jam315, a Japanese pure breed spinning green cocoon and Jam316, a Chinese pure breed spinning white cocoon (Ryu *et al.*, 2013). Red silk (RS) silkworm strain weaving red silk fibers was F1 hybrid between BPred pure breed and Jam157 (unpublished data, S.D.J., H.Y.K., and K.Y.K.). Those color differences were known to be caused by any genetic alteration in signal transduction pathways transporting carotenoids from the midgut lumen to the silk gland (Tsuchida and Sakudoh, 2015). Different colored silk threads were known to be caused by the accumulation of specific phytochemicals in silk glands, suggesting that silk glands in mature silkworm larvae with 3 different colors might have different nutrition components. Thus, we performed proximate, amino acid composition, mineral, carbohydrate, and fatty acid analysis to examine any difference among 3 silkworm varieties.

Materials and Methods

Silkworm varieties, rearing methods, and steamed and freeze-dried mature larval powder (SMSP) processing protocols

Three silkworm varieties were used in this study. Golden silk (GK)(Kang *et al.*, 2007), Yeonnokjam (YNK)(Kang *et al.*, 2007), and Red silk (RS) silkworms were reared with mulberry leaves at the NIAS campus in Wanju-gun, Jeollabuk-do, Korea. Mature silkworm larval powders were generated as previously published (Ji *et al.*, 2015). Briefly, after steamed at 100°C for 130 min using an electric pressure-free cooking machine (Kum Seong Ltd., Boocheon, Korea), mature silkworm larvae were freeze-dried with a freeze-drier (FDT-8612, Operon Ltd. Kimpo, Korea) at -50°C for 24 h. The freeze-dried silkworm larvae were pulverized twice using a hammer mill (HM001, Korean Pulverizing Machinery Co. LTD., Incheon, Korea) and a disk mill (Disk Mill01, Korean Pulverizing Machinery Co. LTD) sequentially (Ji *et al.*, 2015).

Proximate analysis of SMSP generated from 3 silkworm varieties

The amount of H_2O in SMSPs generated from 3 different colored silkworm varieties were quantified by drying at 105°C under atmospheric pressure. The crude lipid in the dried SMSPs were extracted by diethyl ether and then analyzed using a Soxhlet extraction system (Soxtec System HT1043 extraction unit, Foss Tectator). The content of crude fibers in the leftovers from the diethyl ether extractions was quantified by 1.25 % H_2SO_4 and 1.25 % NaOH digestion methods. The content of crude ash was quantified by a dry ashing method at 600°C. The content of crude protein in 3 different SMSPs was examined by semi-micro-Kjeldahl methods using an automatic protein analyzer (Kjeltec 2400 AUT, Poss Tecator, Mulgrave, Australia).

Amino acid composition analysis of SMSPs

The amino acid contents in the 3 SMSPs were quantified using the protocol in the Korea food code (http://fse.foodnara.go.kr/residue/mobile/menu_01_03.jsp?idx=274) and followed methods published in Ji *et al.* (Ji *et al.*, 2016). Three different quantification solutions were generated. The quantification solution I used for measuring Cysteine (CYS) and Methionine (MET) were generated by mixing equal amounts of SMSPs with 20 mL formic acid. The mixture were left at 4°C for overnight to remove volatile compounds and then mixed with 6 N HCl for protein hydrolysis. The quantification solution II used for measuring all amino acids except CYS, MET, and Tryptophan (TRP) were made by inserting N_2 gas to the samples and 6 N HCl mixture to hydrolysis proteins at 110 ± 1°C for 22 ± 1 h. HCl in the quantification solution I and II were removed by a rotary evaporator and hydrolyzed samples were neutralized with H_2O three times before concentrated by a rotary evaporator. The amino acid contents were measure by an automatic amino acid analyzer (Hitachi L-8900A, Hitachi, Tokyo, Japan) according to the manufacturer's protocol. The quantification solution III for measuring TRP amount was made by mixing SMSPs samples with 20 mL of 4.2 N NaOH. After N_2 gas was added, proteins in mixed samples were hydrolyzed at 110 ± 1°C for 22 ± 1 h. Six N HCl was added to neutralize samples and 0.2 N sodium citrate solution was used to adjust samples to pH 4.25. The amount of TRP was measured by an automatic amino acid analyzer (Hitachi L-8900A) according to the manufacturer's protocol.

Analysis of amounts of minerals in the 3 SMSPs

The amounts of minerals in the 3 SMSPs were measured following the protocol from the Association of Official Analytical Chemist (AOAC, 1990). The pre-incinerated SMPSs in crucibles were completely incinerated at 600°C for 2 h. Before being filtered through No. 6 filter paper (GE Healthcare Life Sciences, Chicago, IL, USA) with hot water, 0.5 g of SMSPs cooled to R.T. was mixed with 10 mL of 50% HCl and incubated overnight. The amounts of minerals in the processed samples were measured using an inductively coupled plasma optical emission spectrometer (PerkinElmer Optima 8300, Perkin-Elmer Corporation, Norwalk, CT, USA) by detecting the wavelength and intensities of specific emitted radiant rays for each mineral.

Analysis of Carbohydrates in the 3 SMSPs

To quantify the amounts of 5 different carbohydrates, such as glucose, fructose, sucrose, maltose, and lactose in 3 SMSPs, they were mixed with 15 mL of 50% ethanol. The samples were vortexed, sonicated for 20 min in an 80°C water bath, and then cooled on ice for 3 min. After being mixed for 15 min with 2,000 rpm in the shaker, the samples were spun down for 10 min at 3,000 rpm. After being filtered by syringe filters (0.2 μm pore size), the samples were analyzed by Shiseido Nanospace SI-2 (Shiseido, Tokyo, Japan) with a refractive index detector using the guard cartridge Unison UK-Amino (5 × 2 mm, 3 μm, Imtakt Corp, Kyoto, Japan) and the analytical column Unison UK-Amino (250 × 3 mm, 3 μm, Imtakt). 90% acetonitrile was used as the mobile phase with a column temperature of 60°C and a flow rate of 0.4 mL/min. No carbohydrate was present in the 3 SMSPs (data not shown).

Analysis of fatty acid contents in the 3 SMSPs

The contents of fatty acids in the 3 SMSPs were determined by the Folch method (Folch *et al.*, 1957). Briefly, 50 g of sample was mixed with 250 mL of reaction solution (chloroform: MeOH = 2:1) and then homogenized to extracts lipids from the 3 SMSPs. To dehydrate the extracted lipids, anhydrous Na_2SO_4 were added and concentrated at 50 ~ 55°C. One mL of tricosanoic acid and 1 mL of 0.5 N NaOH was added, sequentially. The samples were cooled after being boiled for 20 min at 100°C. The samples were mixed with 2 mL of BF3-methanol, heated for 20 min and then allowed to cool to R.T. for 30 min before adding 1 mL of heptane and 8 mL of NaCl were added. The supernatants were analyzed using an Agilent US/HP 6890 (Agilent Technologies) equipped with flame ionization detectors and an Omegawax 205 fused-silica bond capillary column (30 m length × 2 mm internal diameter, 0.25 mm film thickness, Sigma-Aldrich, St. Louis, MO. USA). The mobile phase for this analysis was N_2 (99.99%) with a flow rate of 1 mL/min and a 100:1 split ratio. The temperatures for the oven, injection, and detector were 200°C, 250°C, and 260°C, respectively.

Analysis of cholesterol contents

After mixing 5 grams of the 3 SMSPs with 8 mL of 60% KOH and 40 mL of reaction alcohol (EtOH:MeOH:isopropyl alcohol

= 90:5:5), saponification process was conducted by incubating samples in a 100°C water bath for 1 h followed by cooling to 50°C. The samples were mixed with 60 mL of reaction alcohol and then filtered again. Fifty mL of benzenes and 50 mL of 1N KOH were added to a separatory funnel and then allowed to undergo layer separation for 1 h. After repeating layer separation, 20 mL of 0.5 N KOH was added to supernatant and then incubated for 1 h. The lower-phase solution was discarded and the supernatants were mixed with dH_2O and incubated for 1 h. The lower-phase solution was discarded and the supernatant was washed in dH_2O 3 times until transparent. A rotatory evaporator was used to concentrate the supernatants. Squalene in hexane (0.5164 g/500 mL, 1000 ppm) was used as an internal standard. An Gas chromatograph (Agilent 7890A, Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-1 column (Agilent) was operated at following conditions: an injection temperature of 270°C, oven temperature at 260°C for 5 min and then 280°C for 10 min, and a detector temperature of 290°C.

Statistical analysis

To determine significant differences among 3 SMSPs, a one-way analysis of variance (ANOVA) followed by two sample T-tests were performed using the Excel program (Microsoft, Redmond, WA, USA).

Results

The crude nutrient contents in the SMSPs from 3 different silkworm varieties

The crude nutrient compositions of 3 SMSPs were analyzed by performing proximate analyses (Fig. 1). The amounts of H_2O and ash in the GS-SMSP were significantly different from those of YNJ- or RS-SMSPs. The amount of H_2O in GS-SMSP (4.02 ± 0.015 %) was ~ 20% more than those of YNJ-SMSP (2.96 ± 0.05 %, $p < 0.05$) or RS-SMSP (3.28 ± 0.025 %, $p < 0.05$). The amount of ash in GS-SMSP (4.15 ± 0.025 %) was 14.0 % or 8.0 % more than those of YNJ-SMSP (3.57 ± 0.02 %, $p < 0.05$) or RS-SMSP (3.82 ± 0.025 %, $p < 0.05$). There was no significant difference in the crude lipid among 3 SMSPs. The amounts of crude lipids in 3 SMSPs ranged from 11.37 ± 0.15 % to 12.24 ± 0.14 %. The amounts of crude fibers in 3 SMSPs were

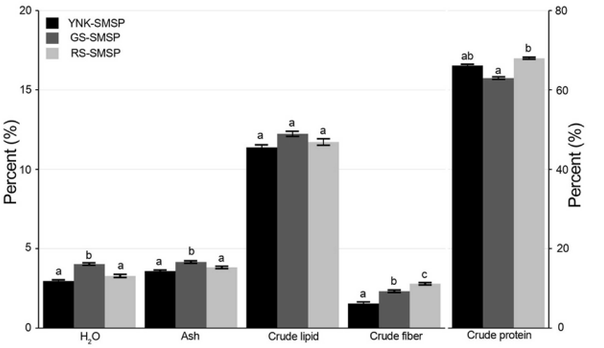

Fig. 1. The proximate analysis of SMPSs making 3 different colored cocoons. Yeonnokjam (YNJ) The contents of H_2O, ash, crude lipid, crude fiber and crude protein were measured. Golden silk (GS)-SMSP had significantly more H_2O and ash than other SMSPs. There was no difference in crude lipids among 3 SMSPs. Red silk (RS)-SMSP had more crude fiber than other SMSPs. GS-SMSP had more crude protein than GS-SMSP. Samples were analyzed by a one-way ANOVA followed by a 2-sample T-Test. a, b, c Different letters indicate significant differences at $p < 0.05$.

significantly different each other. The amount of the crude fiber in YNJ-SMSP (1.54 ± 0.075 %) was significantly less than those of GS-SMSP (2.32 ± 0.001 %, $p < 0.001$) or RS-SMSP (2.78 + 0.01 %, $p < 0.001$). The amount of the crude protein in GS-SMSP (63.02 ± 0.185 %) was significantly less than those of RS-SMSP (67.99 ± 0.01 %, $p < 0.05$), but similar to those of YNJ-SMSP (66.12 + 0.075 %, $p > 0.05$). The most abundant crude components in 3 SMSPs were crude proteins (Fig. 1).

More amino acids in RS-SMSP than YNJ- or GS-SMSP

Among 18 amino acids examined in this study, the amounts of Aspartic acid (ASP), Threonine (THR), Iso-leucine (ILE), Histamine (HIS), Arginine (ARG), Proline (PRO), and TRP in 3 SMSPs did not show any significant difference (Table 1). The amount of CYS in RS-SMSP (0.403 ± 0.004 %) was significantly less than those of GS-SMSP (0.432 ± 0.0002 %, $p < 0.05$), but similar to those of YNJ-SMSP (0.461 ± 0.013 %, $p > 0.05$). The amount of MET in YNJ-SMSP (0.653 ± 0.006 %) was significantly less than those of in GS-SMSP (0.712 ± 0.004 %, $p < 0.05$) or RS-SMSP (0.739 ± 0.001 %, $p < 0.05$). The amount of Serine (SER) in GS-SMSP (5.749 ± 0.069 %) was significantly less than that in YNJ-SMSP (6.498 ± 0.024 %, $p < 0.05$) or RS-SMSP (6.448 ± 0.03 %, $p < 0.05$). The amount of GLU in YNJ-SMSP (3.971 ± 0.007 %) was similar to that in GS-SMSP (4.168

Table 1. Amino acid contents in the 3 SMSPs (mean ± STDE, %)

Amino acids	Silkworm varieties		
	Yeonnokjam	Golden silk	Red silk
Cysteine (CYS)	0.461 ± 0.013^{ab}	0.432 ± 0.0002^{a}	0.403 ± 0.004^{b}
Methionine (MET)	0.653 ± 0.006^{a}	0.712 ± 0.004^{b}	0.739 ± 0.001^{b}
Aspartic acid (ASP)	4.632 ± 0.011^{a}	4.596 ± 0.054^{a}	4.752 ± 0.017^{a}
Threonine (THR)	2.177 ± 0.004^{a}	2.137 ± 0.027^{a}	2.150 ± 0.008^{a}
Serine (SER)	6.498 ± 0.024^{a}	5.749 ± 0.069^{b}	6.448 ± 0.03^{a}
Glutamic acid (GLU)	3.971 ± 0.007^{a}	4.168 ± 0.040^{ab}	4.336 ± 0.016^{b}
Glycine (GLY)	11.006 ± 0.068^{a}	9.468 ± 0.096^{b}	11.552 ± 0.051^{c}
Alanine (ALA)	8.861 ± 0.051^{a}	7.722 ± 0.075^{b}	9.284 ± 0.039^{c}
Valine (VAL)	2.350 ± 0.005^{ab}	2.296 ± 0.039^{a}	2.419 ± 0.006^{b}
Isoleucine (ILE)	1.168 ± 0.001^{a}	1.238 ± 0.024^{a}	1.209 ± 0.0001^{1}
Leucine (LEU)	2.030 ± 0.004^{a}	2.158 ± 0.028^{ab}	2.131 ± 0.006^{b}
Tyrosine (TYR)	4.232 ± 0.016^{a}	3.926 ± 0.044^{a}	4.416 ± 0.016^{b}
Phenylalanine (PHE)	1.692 ± 0.001^{a}	1.785 ± 0.019^{ab}	1.913 ± 0.004^{b}
Lysine (LYS)	2.281 ± 0.004^{a}	2.382 ± 0.048^{ab}	2.518 ± 0.019^{b}
Histidine (HIS)	1.141 ± 0.005^{a}	1.136 ± 0.021^{a}	1.114 ± 0.008^{a}
Arginine (ARG)	2.026 ± 0.012^{a}	2.045 ± 0.054^{a}	2.061 ± 0.014^{a}
Proline (PRO)	1.213 ± 0.007^{a}	1.239 ± 0.033^{a}	1.259 ± 0.01^{a}
Tryptophan (TRP)	0.431 ± 0.01^{a}	0.445 ± 0.003^{a}	0.435 ± 0.007^{a}
Total	56.82 ± 0.209^{a}	53.634 ± 0.961^{b}	59.14 ± 0.331^{c}

Samples were analyzed by a one-way ANOVA followed by a two-sample t-Test.
a, b, c Different letters indicate significant differences at $p < 0.05$.

± 0.040 %, $p > 0.05$), but less than that in RS-SMSP (4.336 ± 0.016 %, $p < 0.05$). The amount of Glycine (GLY) in RS-SMSP (11.552 ± 0.051 %) was significantly more than that in YNJ-SMSP (11.006 ± 0.068 %, $p < 0.05$) or RS-SMSP (9.468 ± 0.096 %, $p < 0.05$). The amount of Alanine (ALA) in GS-SMSP (7.722 ± 0.075 %) was significantly less than that in YNJ-SMAP (8.861 ± 0.051 %, $p < 0.05$) or RS-SMSP (9.284 ± 0.039 %, $p < 0.05$). The amount of Valine (VAL) in GS-SMSP (2.296 ± 0.039 %) was similar to that in YNJ-SMSP (2.350 ± 0.005 %, $p > 0.05$), but significantly less than that in RS-SMSP (2.419 ± 0.006 %, $p < 0.05$). The amount of Leucine (LEU) in YNJ-SMSP (2.030 ± 0.004 %) was similar to that in GS-SMSP (2.158 ± 0.028 %, $p > 0.05$), but significantly less than that in RS-SMSP (2.131 ± 0.006 %, $p < 0.05$). The amount of Tyrosine (TYR) in RS-SMSP (4.232 ± 0.016 %) was significantly more than that in YNJ-SMSP (3.926 ± 0.044 %, $p < 0.05$) or GS-SMSP (4.416 ± 0.016 %, $p < 0.05$). The amount of Phenylalanine (PHE) in RS-SMSP (1.913 ± 0.004 %) was similar to that in GS-SMSP (1.785 ± 0.019 %, $p > 0.05$), but significantly more than that in YNJ-SMSP (1.692 ± 0.001 %, $p < 0.05$). The amount of Lysine (LYS) in RS-SMSP (2.518 ± 0.019 %) was similar to that in GS-SMSP (2.382 ± 0.048 %, $p > 0.05$), but significantly more than that in YNJ-SMSP (2.281 ± 0.004 %, $p < 0.05$). The total amounts of detected amino acids in RS-SMSP (59.14 ± 0.331 %) were significantly more than that in YNJ-SMSP (56.82 ± 0.209 %, $p < 0.05$) or GS-SMSP (53.634 ±0.961 %, $p < 0.05$).

Essential and rare minerals enriched in 3 SMSPs

The contents of essential and rare minerals in 3 SMSPs were compared. Minerals in 3 SMSPs could be divided into 3 groups. The 1st groups presented more than 100mg/100g samples were calcium (Ca), potassium (K), magnesium (Mg), phosphorus (P), and sulfur (S). Similar to proximate analysis results, GS-

Table 2. The amounts of essential and rare minerals in the 3 SMSPs (Mean ± STDE, mg/100g)

Minerals	Silkworm varieties		
	Yeonnokjam	Golden silk	Red silk
Calcium (Ca)	206.9 ± 2.09[a]	213.2 ± 2.48[b]	249.1 ± 12.1[ab]
Potassium (K)	1110.8 ± 13.7[a]	1412.9 ± 6.39[b]	1088.9 ± 59.9[a]
Magnesium (Mg)	211.4 ± 2.74[a]	218.2 ± 0.27[a]	206.6 ± 10.3[a]
Phosphorus (P)	702.9 ± 2.25[a]	811.7 ± 9.21[b]	633.2 ± 34.95[a]
Sulfur (S)	323.3 ± 3.91[a]	299.2 ± 8.01[a]	250.2 ± 1.58[b]
Copper (Cu)	1.936 ± 0.110[a]	2.032 ± 0.025[a]	2.059 ± 0.015[a]
Iron (Fe)	3.664 ± 0.121[a]	3.443 ± 0.673[a]	5.454 ± 0.036[b]
Sodium (Na)	14.03 ± 2.686[a]	14.46 ± 0.465[a]	12.20 ± 1.48[a]
Manganese (Mn)	1.598 ± 0.005[a]	4.403 ± 0.005[b]	1.697 ± 0.005[c]
Zinc (Zn)	5.429 ± 0.025[a]	5.211 ± 0.077[a]	5.049 ± 0.282[a]
Chromium (Cr)	0	0	0
Lead (Pb)	0	0	0
Cadmium (Cd)	0	0	0
Mercury (Hg)	0	0	0
Arsenic (As)	0	0	0

Samples were analyzed by a one-way ANOVA followed by a 2-sample T-Test.
a, b, c Different letters indicate significant differences at $p < 0.05$.

SMSP contained more minerals than other SMSPs with some exceptions. For examples, the amount of K in GS-SMSP (1412.9 ± 6.39 mg/100 g) was ~ 30% more than that in YNJ-SMSP (1110.8 ± 13.7 mg/100 g, $p < 0.05$) or RS-SMSP (1088.9 ± 59.9 mg/100 g, $p < 0.05$). The amount of P in GS-SMSP (811.7 ± 9.21 mg/100 g) was 15.5% or 28.1% more than that of YNJ-SMSP (702.9 ± 2.25 mg/100 g, $p < 0.05$) or RS-SMSP (633.2 ± 34.95 mg/100 g, $p < 0.05$), respectively. In case of Ca, however, RS-SMSP (249.1 ± 12.1 mg/100g) had 20.1 % or 16.9 % more than that in YNJ-SMSP (206.9 ± 2.09 mg/100 g, $p < 0.05$) or GS-SMSPs (213.2 ± 2.48 mg/100 g, $p > 0.05$). There was no differences in the amounts of Mg among 3 SMSPs. Interestingly, the amount of S in YNJ-SMSP (323.3 ± 3.91 mg/100 g) were 8.0% or 29.2% more than that of GS-SMSP (299.2 ± 8.01 mg/100 g, $p > 0.05$) or RS-SMSP (250.2 ± 1.58 mg/100 g, $p < 0.05$), respectively.

The 2nd groups presented between 1 to 20 mg/100g samples were included copper (Cu), iron (Fe), sodium (Na), manganese (Mn) and zinc (Zn). The amounts of Cu, Na, and Zn in 3 SMSPs were not significantly different each other. Interestingly, the amount of Mn in GS-SMSP (4.403 ± 0.005 mg/100 g) was 2.75 or 2.60 times more than that in YNJ-SMSP (1.598 ± 0.005 mg/100 g, $p < 0.05$) or RS-SMSP (1.697 ± 0.005 mg/100 g, $p < 0.05$), respectively. In addition, the amount of Fe in RS-SMSP (5.454 ± 0.036 mg/100 g) was significantly more than that in YNJ-SMSP (3.664 ± 0.121 mg/100 g, $p < 0.05$) or GS-SMSP (3.443 ± 0.673 mg/100g, $p < 0.05$).

The 3rd group was heavy metals such as lead, cadmium, chromium, mercury, and arsenics which were not presented in 3 SMSPs. These results suggested that 3 SMSPs were enriched with essential and rear minerals but not contaminated with toxic heavy metals.

High ratios of unsaturated fatty acids in 3 SMSPs

We further analyzed whether there were differences in 14 fatty acids among 3 SMSPs (Table 3), because crude lipids were the 2nd most common crude components in SMSPs (Fig. 1). Among 14 fatty acids, Vaccenic acid, Eicosatrienoic acid, Eicosapentaenoic acid, Docosatetraenoic acid, and Docosahexaenoic acid were not detected in 3 SMSPs (Table 3). Palmitic acid was the most abundant saturated fatty acids in 3 SMSPs (YNJ; 23.64 %, GS; 24.10 %, and RS; 23.71 %) among 3 detected saturated fatty acids (Table 3). The amounts of total

Table 3. Fatty acid compositions of the 3 SMSPs (content, % of total fatty acids)

Fatty acids	Content (% of total fatty acids)		
	Silkworm varieties		
	Yeonnokjam	Golden silk	Red silk
Palmitic acid (C16:0)	23.64	24.10	23.71
Stearic acid (C18:0)	8.42	9.13	7.64
Myristic acid (C14:0)	0.15	0.18	0.19
Oleic acid (C18:1n-9)	33.73	29.54	24.44
Linolenic acid (C18:3n-3)	26.79	29.08	36.68
Linoleic acid (C18:2n-6)	6.04	6.84	6.16
Palmitoleic acid (C16:1n-7)	0.82	0.75	0.72
Eicosenoic acid (C20:1n-9)	0.31	0.25	0.31
γ-Linoleic acid (C18:3n-6)	0.10	0.12	0.16
Vaccenic acid (C18:1n-7)	0.00	0.00	0.00
Eicosatrienoic acid (C20:3n-3)	0.00	0.00	0.00
Eicosapentaenoic acid (C20:5n-3)	0.00	0.00	0.00
Docosatetraenoic acid (C22:4n-6)	0.00	0.00	0.00
Docosahexaenoic acid (C22:6n-3)	0.00	0.00	0.00
Total	100.00	99.99	100.01
∑Saturated fatty acids	32.21	33.41	31.54
∑Unsaturated fatty acids	67.79	66.58	68.47
∑Mono-unsaturated	34.86	30.54	25.47
∑Poly-unsaturated	32.93	36.04	43.00
n-6/n-3	0.23	0.24	0.17

saturated fatty acids were 31.54 % (RS-SMSP), 32.21 % (YNJ-SMSP), and 33.41 % (GS-SMSP).

Oleic acid was the most abundant unsaturated fatty acids among 6 unsaturated fatty acids detected from 3 SMSPs. The ratio of Oleic acid in YNJ-SMSP (33.73 %) was higher than that in GS-SMSP (29.54 %) or RS-SMSP (24.44 %). The ratio of Linolenic acid, a Ω-3 (n-3) fatty acid in RS-SMSP (36.68 %) was higher than that in YNJ-SMSP (26.79 %) or GS-SMSP (29.08 %). The ratios of Linoleic acid among 3 SMSPs were similar, ranged from 6.04 % to 6.84 %. The other 3 low abundant unsaturated fatty acids, such as Palmitoleic acid, Eicosenoic acid, and γ-Linoleic acid were also similarly presented in 3 SMSPs (Table 3)

Although the total amount of unsaturated fatty acids among 3 SMSPs were similar (ranged from 66.58 % to 68.47 %), the total amount of mono- or poly-unsaturated in 3 SMSPs were different each other. The total mono-saturated fatty acids in YNJ-SMSP

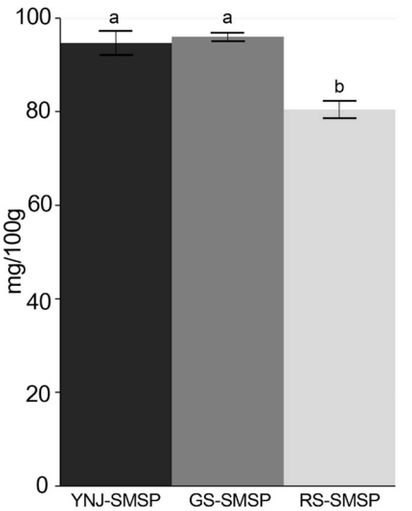

Fig. 2. The amounts of cholesterols in 3 SMSPs.
The content of cholesterol in RS-SMSP was significantly less than that in YNJ- and GS-SMSPs.
Samples were analyzed by a one-way ANOVA followed by a 2-sample T-Test. a, b. Different letters indicate significant differences at $p < 0.05$.

(34.86 %) appeared to be higher than that in GS-SMSP (30.54 %) or RS-SMSP (25.47 %). The total poly-unsaturated fatty acids in RS-SMSP (43.00 %) appeared to be higher than that in YNJ-SMSP (32.93 %) or GS-SMSP (36.04 %). In addition, the ratio of n-6/n-3 fatty acids in RS-SMSP (0.17) appeared to be lower than that in YNJ-SMSP (0.23) or GS-SMSP (0.24).

Cholesterol amounts in 3 SMSPs

The amount of cholesterol in RS-SMSP (80.45 ± 1.825 mg/100 g) was significantly more than that in YNJ-SMSP (94.72 ± 2.53 mg/100 g, $p < 0.05$) or GS-SMSP (95.98 ± 1.83 mg/100 g, $p < 0.05$).

Discussion

In previous studies we have shown that SMSP made from white jade silkworm (also known as Back Ok Jam) (Lee *et al.*, 1984) had different crude nutrients and amino acid compositions than FDSP (Ji *et al.*, 2016) which has been known to have hypoglycemic effects in animals and humans (Ryu *et al.*, 2012; Ryu *et al.*, 2013). We also reported that SMSP has several health improvements effects such as extended healthspan and increased resistances to Parkinson's disease (Nguyen *et al.*, 2016). Those differences in health improvements effects between FDSP and SMSP might be caused by different nutrient compositions.

Using total 340 silkworm pure breeds secured in NIAS, we have generated silkworm varieties which had superior traits including cocoon colors and sizes, fecundity etc. Among them, YNJ-, GS-, and RS-silkworms have unique cocoon colors with reliable cocoon sizes and fecundity (Kang *et al.*, 2007; Ryu *et al.*, 2013). Studies in cocoon colors in silkworm pupae have shown that any defect in signal transduction pathways transporting phytochemicals from midgut lumens to silk glands contributed in different colors, although all silkworms were fed with the same mulberry leaves (Tsuchida and Sakudoh, 2015). In addition, it has been shown that mulberry leaves contained various phytochemicals such as flavonoids, polyphenols, carotenoids etc. (Iqbal *et al.*, 2012). Various studies have shown that natural phytochemicals in mulberry leaves could have various health improvement effects such as hypoglycemic (Mudra *et al.*, 2007), hypolipidemic (Aramwit *et al.*, 2011), antihypertensive effects (Yang *et al.*, 2012), and anti-tumor activity (Fathy *et al.*, 2013).

Recently we have shown that hypoglycemic effect of FDSP, the most widely consumed silkworm products in Korea might be originated from 1-Deoxynojirimycin from mulberry leaves (Ryu *et al.*, 2013). In addition to causing different cocoon colors, plethora of phytochemicals in mulberry leaves could give some effects in physiologies of silkworm varieties, if they were not properly transports, metabolized, or secreted. Thus, silkworms making cocoons with different colors might have different health improvement effects. Thus, we examined the nutrient components in 3 SMSPs. Although crude nutrient components in 3 SMSPs were quite similar (Fig. 1, Table 1, 2, and 3), each SMSP had unique characteristics. For examples, the crude proteins and amino acid components in RS-SMSPs were significantly more than the other SMSPs (Fig. 1 and Table 1). Especially amounts of SER, GLU, GLY and ALA in RS-SMSPs were equal or significantly more than those in YNJ- or GS-SMSPs.

The proximate analysis showed that the amount of ash in GS-SMSP was more than that in YNJ- and RS-SMSPs (Fig. 1). Consistent with this result, contents of several minerals in GS-SMSP were significantly more than that of YNJ- and RS-SMSPs. Since it has been reported that uptake of foods containing more K and less Na could reduce blood pressure in humans (Aburto *et al.*, 2013) and GS-SMSP (97.71) had the highest K/NA ratio compared to that of YNJ- (79.19) and RS-SMSP (89.25) (Table 2), it could be reduced blood pressures in humans.

The ratio of poly- to mono-unsaturated fatty acids in RS-SMSP appeared to be higher than YNJ- and GS-SMSPs (Table 3). Although there are still controversy regarding what kinds of unsaturated fatty acids could have more health benefits in humans, it has been suggested that the ratio of n-6/n-3 unsaturated fatty acids in foods was one of important factors affecting risks of metabolic disorders (Simopoulos, 2002). Most of westernized fast foods had high n-6/n-3 fatty acid ratio in addition to high saturated fatty acid contents, possibly increasing numbers of patients with metabolic disorders in developed countries. Therefore, 3 SMSPs examined in this study could be excellent health supplements for providing n-3 unsaturated fatty acids and RS-SMSP might be the best choice.

Taken together, our data suggested that the 3 SMSPs might have common and unique health improvement effects in humans. Thus, further studies in the functionalities of the 3 SMSPs will reveal unknown their health improvements effects.

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