

Comparison of silkworm powder from 3 *Bombyx mori* varieties on alcohol metabolism in rats

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Abstract

Increased alcohol consumption is a burden on the world because it is associated with various health problems. However, the effects of silkworms on alcohol metabolism have not been studied yet. The hard-to-eat mature silkworms have become easier to ingest recently due to the development of technology, steam-lyophilising mature silkworm larvae. In this study, we investigated and compared the effects of SMSPs from three silkworm varieties, Baekokjam, Golden-silk and Yeonnokjam weaving white, golden, and light green cocoons on alcohol metabolism *in vivo*. Sprague-Dawley rats pretreated with three SMSPs (0.1 g/kg or 1 g/kg body weight) or normal diet (AIN-76A) for 2 weeks were subjected to intragastric administration of absolute ethanol (3 g/kg body weight, 3 h). Three SMSPs did not affect the final body weight and liver weight. All 3 SMSPs were effective to reduce the enzymes in alcohol metabolism, alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH), and liver damage and enzymes involved in liver damage, aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Among SMSP from 3 varieties of silkworm, preadministration of 1 g/kg Baekokjam SMSP showed the most effective suppressive effect on the activities of ADH, ALDH, AST and ALT. The Baekokjam SMSP contained higher amounts of beneficial amino acids than Golden-silk or Yeonnokjam SMSP. These results suggest that Baekokjam SMSP might be used as a new and promising candidate for improving alcohol metabolism and liver injury through promoting rapid alcohol metabolism.

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Introduction

According to WHO report published in 2014, worldwide people consumed 6.2 liters of alcohol in average in 2010. The highest consumption has been reported in eastern and central Europe and sub-saharan Africa (Shield *et al.*, 2013).

However, it is well known that South Koreans drink a lot of alcohol, even to that South Koreans drink twice as much liquor as Russians and more than four times as much as Americans. Drinking alcohol beverages has been linked to a large number of health disabilities, including diabetes mellitus, neuropsychiatric disorders, cardiovascular disease,

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cirrhosis of the liver, and cancer (Scoccianti *et al.*, 2014). The major enzymes of alcohol metabolism and its pathway are alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH) (Cequera *et al.*, 2014). These enzymes are known as phase I xenobiotic metabolizing enzymes, which perform important roles in reduction of alcohol (Cequera *et al.*, 2014). ADH rapidly oxidizes alcohol to acetaldehyde, and ALDH converts acetaldehyde to acetate (Park *et al.*, 2012). Acute heavy drinking can cause hangover symptoms due to acetaldehyde, which is known to be a primary metabolite of alcohol (Jung *et al.*, 2016). Therefore, development of new agent targeting alcohol metabolism may be a strategy to control the complications of alcohol consumption (Sung *et al.*, 2012).

The silkworm, *Bombyx mori*, a major species for sericulture, has been cultivated since ancient times. Traditionally, the applications for silkworm byproducts have been limited to making fabrics using silk from cocoons (Cho *et al.*, 2016). Silkworms, which were difficult to eat due to the large silk gland, have recently become easier to eat due to the discovery of technique for processing mature silkworm larvae to steamed and freeze-dried mature silkworm larval powder (SMSP) (Ji *et al.*, 2015). SMSP has been reported to contain useful proteins, high amounts of amino acids and essential minerals (Ji *et al.*, 2016). Several silkworm varieties have been developed in National Institute of Agricultural Science (NIAS), which have various phenotypes such as colors and sizes of cocoons (Kang *et al.*, 2007; Ryu *et al.*, 2013). Our colleagues have demonstrated that nutrient compositions of SMSP were varied depending on the varieties of silkworm (Ji *et al.*, 2016). In recent years, silkworms have been using as producing functional foods with various health improvement effects (Ji *et al.*, 2016). The silkworm has been reported to have the effect of preventing hyperlipidemia and hyperglycemia in rats (Kim *et al.*, 2008). The silk fibroin hydrolysate has been reported to control blood glucose concentrations in mice (Do *et al.*, 2012). Additionally, silk fiber from silkworm has reported to decrease the levels of blood cholesterol, glucose and alcohol absorption (Kunz *et al.*, 2016). However, the effects of silkworms on alcohol metabolism have not been studied yet. Thus, we investigated and compared the effects of SMSPs from three silkworm varieties which were weaving white, golden, and light green cocoons on alcohol metabolism *in vivo*.

Materials & Methods

Steamed and freeze-dried mature silkworm larval powder (SMSP) Production

Three silkworm varieties, Baekokjam (Lee *et al.*, 1984), Golden-silk (Kang *et al.*, 2007), Yeonnokjam (Kang *et al.*, 2007), were used in the present study. Silkworm larvae (*Bombyx mori*) were reared with mulberry leaves at the National Institute of Agricultural Science. SMSP was made as previously published (Ji *et al.*, 2015). Briefly, live mature larvae of the 3 cocoon strains were immediately smothered for 130 min at 100 °C with steam using an electric pressure-free cooking machine (KumSeong Ltd., Boocheon, Korea) followed by freeze-drying with a freeze-drier (FDT-8612, Operon Ltd. Kimpo, Korea) for 24 h. Larvae were then grinded using a hammer mill (HM001, Korean Pulverizing Machinery Co. LTD., Incheon, Korea) and a disk mill (Disk Mill01, Korean Pulverizing Machinery Co. LTD). The lengths of particles of SMSP were shorter than 0.1 mm. The SMSP was stored at -50 °C and then used for formulating diet for rat.

The preparation of diets containing SMSP

AIN-76A and SMSP containing AIN-76A diets were purchased from DBL (Umsung, Korea). Briefly, 3 varieties SMSP were mixed with powdered AIN-76A, and then this mixture were dried and prepared as a pellet chow by DBL (Umsung, Korea). Two different amounts of SMSP were added to AIN-76A; low dose (1 g/kg of AIN-76A for the treatment with 0.1 g/kg of rat body weight) and high dose (10 g/kg of AIN-76A for the treatment with 1 g/kg of rat body weight). Two different concentrations of diet were similarly formulated.

Animal and experimental design

All the Sprague-Dawley (SD) rats in the experiment were purchased from Orient bio (Seoul, Korea). SD rats were housed in individual standard cages on a 12 h light/dark cycle in a temperature controlled (24 °C) environment during a week of acclimatization, with *ad libitum* access to water and a rodent chow diet. All animal experiment protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of animal center of the CHA University (reference

number: IACUC 150021). After acclimatization, the SD rats were randomized into eight groups ($n = 7$). Each group was fed an experimental diet: the AIN-76A (Normal and Ethanol group) or diets containing Baekokjam SMSP (0.1 or 1 g/kg), Golden-silk SMSP (0.1 or 1 g/kg), and Yeonnokjam SMSP (0.1 or 1 g/kg). The experimental diets were administered for 2 weeks in the form of pellets. Then, all groups except the Normal group were treated ethanol by oral gavage and after 3 hours all rats were sacrificed.

Blood and Tissue Samples

All rats were sacrificed by taking blood via cardiac puncture under carbon monoxide anesthesia after treatment with ethanol for 3 hours. Blood samples were treated with heparin, and the plasma samples were obtained by centrifuging the blood at 3,000 rpm for 15 min at 4 °C. Livers were removed, and rinsed with phosphate-buffered saline. The liver were frozen in liquid nitrogen and stored at - 80 °C until further use.

Analysis of alcohol metabolizing enzymes

For the determination of alcohol dehydrogenase activity (ADH), aldehyde dehydrogenase activity (ALDH) was measured using commercial kits (Biovision #K787-100 and #K731-100, respectively). Briefly, 50 μ L of serum samples put into 96-well plate and added each reaction mix in the amount required by the protocol. ADH was reacted at 37 °C for 3 minutes and ALDH was reacted at room temperature for 5 minutes. After the reaction was complete, the absorbance was measured at 450 nm. After 1 hour, the absorbance was measured at the same absorbance and calculated according to the protocols.

Serum biochemical parameters of hepatic injury

Hepatic injury was evaluated biochemically by measuring the activities of alanine aminotransferase (ALT/GPT) and aspartate aminotransferase (AST/GOT) in serum using Hitachi automatic analyzer 7600-210 (Hitachi high-technologies corporation, Tokyo, Japan)

Statistical Analysis

Results are expressed as the mean \pm standard deviation. The

statistical significance was analyzed by one-way analysis of variance (ANOVA). Statistical significance was accepted at $P < 0.05$.

Results and Discussion

Comparison of SMSPs from 3 silkworm varieties on alcohol metabolizing enzymes

Hangover is a mental or physical discomfort that manifests symptoms such as vomiting, headache, thirst, and loss of concentration. Typically, hangovers start within hours of drinking alcohol, peak at zero blood alcohol concentration, and hangover symptoms can last for hours (Swift *et al.*, 1998). High concentrations of acetaldehyde are known to cause hangover and acetaldehyde is a substance that binds to proteins and biologically important compounds (Swift *et al.*, 1998). After alcohol consumption, alcohol is mainly metabolized by ADH and ALDH, which are responsible for 80% of alcohol metabolism. Other pathways for alcohol metabolism are known to microsomal ethanol oxidizing system and catalase (Cederbaum, 2012). In this study, we compared the effect of 3 varieties SMSPs on main alcohol metabolizing enzymes, ADH and ALDH and hepatic injury induced by ethanol *in vivo*.

To investigate the protective effect of SMSPs on ethanol metabolism, we used ethanol-treated rat model. Single dose of 3 g/kg ethanol was intragastrically injected into 6-week old SD rat after pretreatment with diet containing Baekokjam, Golden-silk or Yeonnokjam varieties of SMSP for two weeks. At the end of the experiment, the body and liver weights were measured and showed in Figure 1. There was no significant difference in body weight or liver weight (Fig. 1). However, the ratio of liver weight to body weight, the marker of liver damage, was increased in ethanol-treated group, whereas the pretreatment with SMSP decreased the ratio of liver weight to body weight (Fig. 1C). This result is thought to be meaningful though there was no statistical significance because alcohol treatment was as short as 3 hours.

Alcohol is almost insoluble in oils and fats but it can spread to all tissues in the body, by which can pass through biological membranes because it is small molecule. Less than 10% of the absorbed alcohol is directly removed by the lungs, kidneys, and sweat, but the absorption and metabolism of the rest of the alcohol is influenced by individual factors such as sex, age,

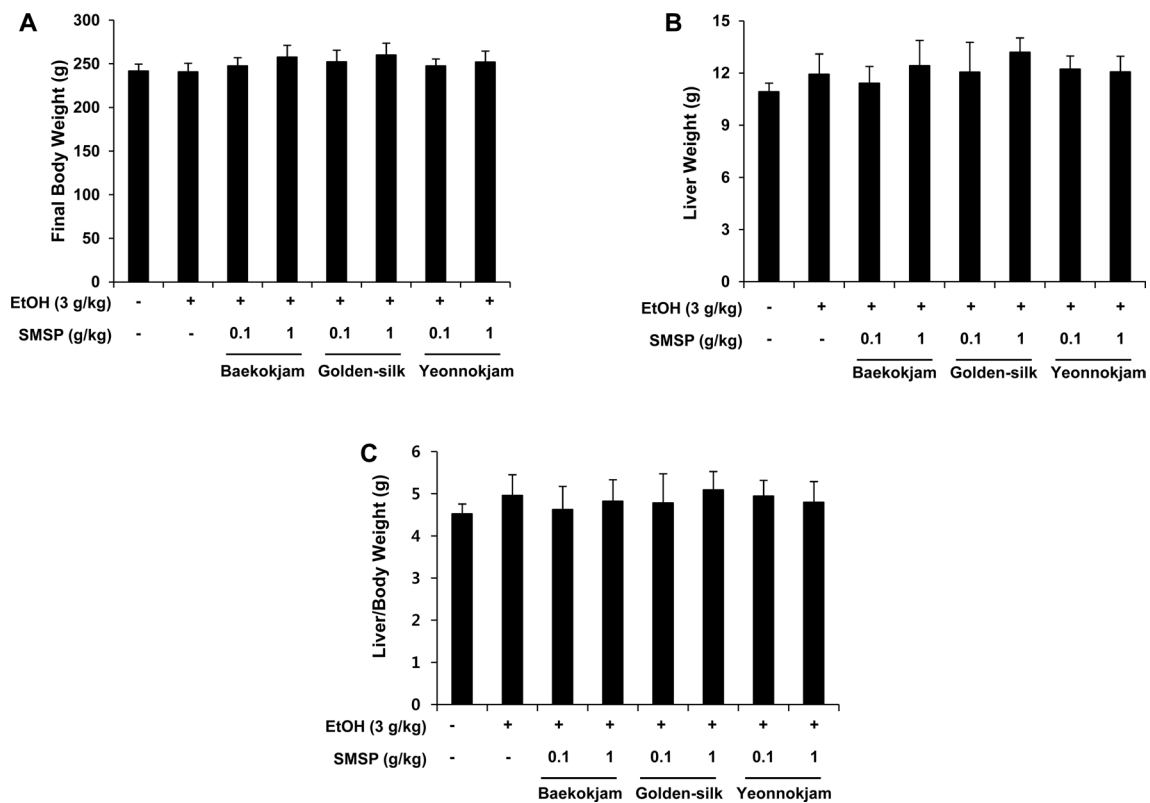


Fig. 1. Measurement of the liver and body weight. The final body weight (A) and liver weight (B) were measured at the end of animal experiment. Pretreatment with 3 kinds of SMSP and ethanol administration did not induce the change of liver and body weight. (C) The ratio of liver weight to the body weight was calculated.

weight, race, and health status (Louvet *et al.*, 2015). Most of the alcohols are oxidized in the liver according to the general mechanism of alcohol oxidation. ADH is an enzyme that contains zinc with two subunits. It is present in the cytosol fraction of the cell and the largest amount is found in the liver. ADH plays a role in oxidizing the alcohol consumed or the alcohol in which the food is digested to acetaldehyde (Cederbaum, 2012). Acetaldehyde is known to be the main cause of hangover and most acetaldehyde is oxidized by ALDH in the liver (Agarwal *et al.*, 1989). ALDH serves not only to oxidize acetaldehyde, but also to remove other aldehydes (Cederbaum, 2012). To study the effect of pretreatment with 3 SMSPs on alcohol metabolism, we treated SMSP-fed rat and normal diet-fed rat with ethanol (3 g/kg) for 3 h. Ethanol-induced the activities of ADH and ALDH was significantly reduced in 3 SMSP-fed rat compared to the normal diet-fed rat (Fig. 2A and B). The activity of ADH in ethanol-treated group (10.87 ± 2.77 mU/mL) was significantly higher than those of 1 g/kg Baekokjam SMSP-fed group (5.26 ± 0.90 mU/mL, $P < 0.01$), 1 g/kg Golden-silk SMSP-fed group

(7.46 ± 3.16 mU/mL, $P < 0.05$), or 1 g/kg Yeonnokjam SMSP-fed group (5.05 ± 1.78 mU/mL, $P < 0.01$). Among 3 varieties of silkworm, Baekokjam SMSP showed the best inhibitory effect on ADH activity induced by ethanol treatment. Similarly, the activity of ALDH (3.55 ± 0.85 mU/mL) was significantly induced by in ethanol treatment, which was decreased by the preadministration of 1 g/kg Baekokjam SMSP-fed group (2.07 ± 0.27 mU/mL, $P < 0.05$), 1 g/kg Golden-silk SMSP-fed group (2.25 ± 0.40 mU/mL, $P < 0.05$), or 1 g/kg Yeonnokjam SMSP-fed group (2.25 ± 0.78 mU/mL, $P < 0.05$). As well as ADH activity, Baekokjam SMSP showed the better inhibitory effect on ALDH activity induced by ethanol treatment than Golden-silk or Yeonnokjam SMSP. These results suggest that all 3 SMSPs, especially Baekokjam SMSP among them can prevent alcohol-induced hangover by regulation of ADH and ALDH.

Most studies have reported that functional foods or compounds relieves ethanol-induced hangover by increasing the activities of ADH and ALDH (Seo *et al.*, 2014). However, pretreatment with 3 SMSPs decreased the activities of ADH and ALDH in this

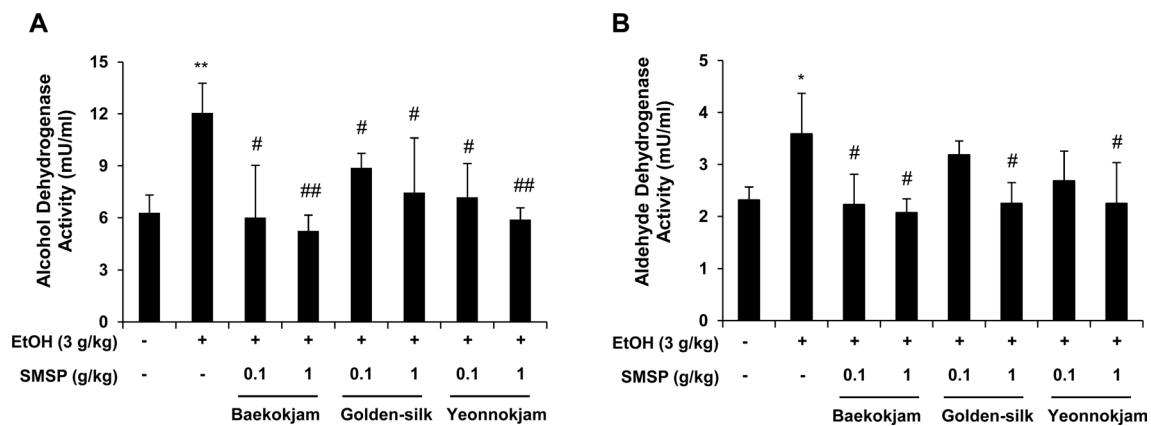


Fig. 2. Comparative analysis of 3 SMSPs on alcohol metabolizing enzymes. The activities of ADH (A) and ALDH (B) were measured in serum. The activities of ADH and ALDH were significantly increased by administration of ethanol, whereas the pretreatment with 3 SMSPs decreased the enzyme activities in rats with ethanol-treated rats. Among 3 kinds of SMSP, Baekokjam strain SMSP showed the best inhibitory effect on alcohol metabolizing enzymes. Results shown as mean \pm SD ($n = 7$). Statistical significance was analyzed by one-way ANOVA. *, $P < 0.05$ and **, $P < 0.01$ (vs Normal group); #, $P < 0.05$ and ##, $P < 0.01$ (vs Ethanol group).

study. Main metabolic enzymes for ethanol are ADH and ALDH, however, which are responsible for only about 80% of alcohol degradation. Other pathways for alcohol metabolism definitely exist (Cederbaum, 2012). Therefore, SMSP can up-regulate other pathways involved in alcohol metabolisms, such as microsomal ethanol oxidizing system or catalase. Another possibility is that SMSP probably enhances the ethanol clearance rate by interfering with the gastrointestinal absorption of ethanol. Recent report demonstrated that the orphan nuclear hormone receptor small heterodimer partner (SHP) deletion increases ethanol catabolism showing decreasing ADH and ALDH activities at 30-60 min after ethanol challenge. Thus, SHP $-/-$ mice exhibited faster blood ethanol clearance and earlier wake-up time (Park *et al.*, 2016). In support of this notion, our previous results showed that SMSP treatment significantly decreased the levels of blood alcohol and acetaldehyde concentration (data not shown). In addition, increased activity of ADH is known to be associated with ROS formation (Mattia *et al.*, 1993). Tahir and Sultana reported that the flavonoid chrysin significantly suppressed the activities of CYP2E1 and ADH involved in the catabolism of ethanol in the liver and kidney when compared with the ethanol-administered rats, thereby reducing ROS-mediated tissue injuries during ethanol administration (Tahir *et al.*, 2011). Consistent with this report, we also observed that SMSP treatment enhances antioxidant capacity and inhibits oxidative stress (data not shown). For these reasons, the inhibitory effects of SMSPs on the activities of ADH and ALDH can be explained.

Comparison of SMSPs from 3 silkworm varieties on acute liver damage induced by ethanol in rats

To determine the ethanol-induced liver damage, the levels of indicators in the serum, AST and ALT were measured by the colorimetric method. ALT/GPT is almost exclusively found in the liver. When the liver tissue is diseased or damaged, additional AST and ALT are released into the bloodstream, which increases their activities. Thus, measuring serum levels of AST or ALT is a valuable tool in the diagnosis of liver damage (Mathews *et al.*, 2014). The activity of AST (126.75 ± 11.14 IU/L, $P < 0.01$) was significantly induced in ethanol-treated rats, which reflect liver damage, whereas significantly suppressed by pretreatment with 1 g/kg Baekokjam SMSP-fed group (97.75 ± 11.58 IU/L, $P < 0.01$), 1 g/kg Golden-silk SMSP-fed group (99.4 ± 12.54 IU/L, $P < 0.01$), or 1 g/kg Yeonnokjam SMSP-fed group (111.00 ± 3.91 IU/L, $P < 0.05$) (Fig. 3A). In addition, the activity of ALT (61.20 ± 6.21 IU/L, $P < 0.01$) was also significantly induced in ethanol-treated rats, which was significantly suppressed by pretreatment with 1 g/kg Baekokjam SMSP-fed group (49.14 ± 2.53 IU/L, $P < 0.01$), 1 g/kg Golden-silk SMSP-fed group (54.33 ± 7.54 IU/L, $P < 0.05$), or 1 g/kg Yeonnokjam SMSP-fed group (54.71 ± 9.72 IU/L) (Fig. 3B). Among SMSP from 3 varieties of silkworm, preadministration of 1 g/kg Baekokjam SMSP showed the most effective suppressive effect on AST and ALT activities (Fig. 3). These results demonstrate that SMSP, especially Baekokjam SMSP pretreatment may have protective effects on ethanol-

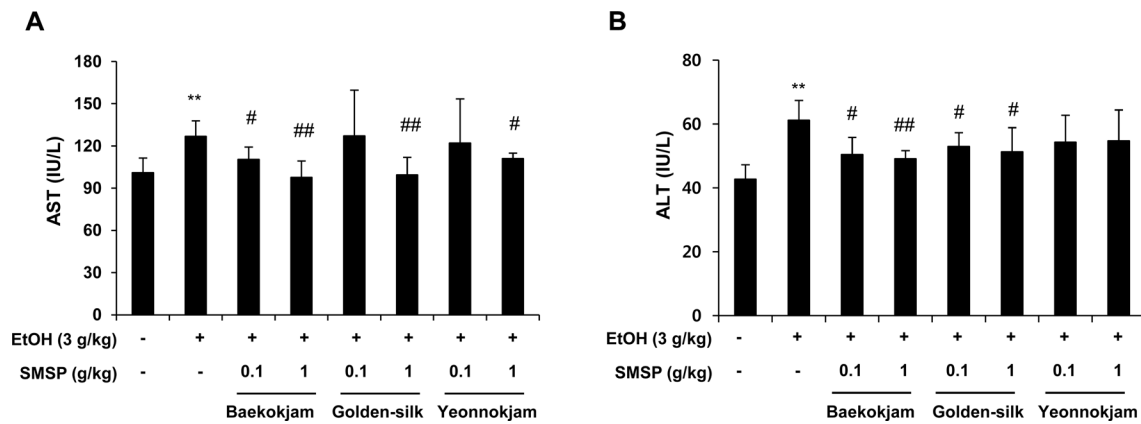


Fig. 3. Measurement of AST and ALT. Plasma concentration of AST (A) and ALT (B) was measured. The activities of AST and ALT were significantly increased by administration of ethanol, whereas the pretreatment with 3 SMSPs decreased the enzyme activities in rats with ethanol-treated rats. Among 3 kinds of SMSP, Baekokjam strain SMSP showed the best inhibitory effect on the levels of AST and ALT. Data shown as mean \pm SD ($n = 7$). Statistical significance was analyzed by one-way ANOVA. **, $P < 0.01$ (vs Normal group); #, $P < 0.05$ and ##, $P < 0.01$ (vs Ethanol group).

Table 1. Composition of amino acids in 3 varieties of SMSP

	Silkworm varieties		
	Baekokjam	Golden-silk	Yeonnokjam
Crude protein (g/100 g)	68.66	66.12	63.02
Amino acids (%)			
Glycine	12.335	9.468	11.006
Alanine	9.778	7.722	8.861
Serine	6.757	5.749	6.498
Aspartic acid	4.679	4.596	4.632
Tyrosine	4.560	3.926	4.232
Glutamic acid	3.824	4.168	3.971
Valine	2.399	2.296	2.350
Lysine	2.193	2.382	2.281
Threonine	2.185	2.137	2.177
Leucine	1.983	2.158	2.030
Arginine	1.958	2.045	2.026
Phenylalanine	1.746	1.785	1.692
Isoleucine	1.214	1.238	1.168
Proline	1.148	1.239	1.213
Histidine	1.083	1.136	1.141
Methionine	0.644	0.712	0.653
Cysteine	0.427	0.432	0.461
Tryptophane	0.425	0.445	0.431

induced hepatic injury.

The NIAS has developed several silkworm varieties by crossing hundreds of silkworm varieties with various phenotypes. These color differences are caused by genetic modifications that carry carotenoids from the central lumen to the silk line during signal transduction. The accumulation of certain plant chemicals in silk glands results in silk threads of different colors, and silkworm mature larvae may have different nutrients depending on their color (Kang *et al.*, 2007; Ryu *et al.*, 2013). The Baekokjam, Golden-silk, and Yeonnokjam SMSPs used in this experiment contain large amounts of crude protein and amino acids that are helpful for health, especially glycine, alanine, serine, aspartic acid and tyrosine (Table 1) (Ji *et al.*, 2016). The amounts of glycine, alanine, serine, aspartic acid and tyrosine in Baekokjam SMSP were higher than those in Golden-silk or Yeonnokjam SMSP (Table 1). It is well known that amino acids such as glycine, alanine, serine, aspartic acid and tyrosine support liver health and energy metabolism, which explains how amino acids-rich SMSP exhibits protective effects on ethanol-induced hepatic injury. Moreover, previous study demonstrated that SMSP contained essential minerals and a large amount of unsaturated fatty acids such as linolenic acid (Ji *et al.*, 2016; Ji *et al.*, 2016).

In conclusion, our results suggest that 3 SMSPs effectively improves alcoholic hangover through regulation of alcohol metabolizing enzymes and protection of liver damage induced by ethanol treatment *in vivo* model. Among SMSPs from 3 varieties of silkworm used in this study, Baekokjam SMSP might

be used as a new and promising candidate for improving alcohol metabolism and hangover relief, as showing the best suppressive effect on alcohol-induced damages.

Conflict of Interests

All authors have nothing to disclose and have no commercial or financial interest in the products described in this paper.

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