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Antioxidant capacity of silkworm pupa according to extraction condition, variety, pupation time, and sex

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Abstract

Silkworm pupa has been used as an edible insect with the high quality of protein and unsaturated fatty acids. In this study, antioxidant activities of pupa according to variety, pupation day, sex, and extraction solvent were analyzed. The 30% ethanol extract showed highest radical scavenging activity compared with the DW, hexane, and 70-100% ethanol extracts. In the DPPH and ABTS radical scavenging assay according to the type of pupa, the antioxidant effect was increased in female with the early stage of pupation day. In cell-based assay, reactive oxygen species (ROS) level was decreased in pupa groups by -30 to -50% followed by *tert*-butyl hydroperoxide (*t*-BHP) treatment. The ROS levels were significantly reduced in 7th day in each variety. In conclusion, the free radical and ROS scavenging effects were increased in female pupa with the early pupation day. The result could be used for development of bioactive materials using silkworm pupa.

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Introduction

The sericulture industry has produced silkworm cocoons, a source of natural fiber, for over five thousand years (Hwang *et al*., 2001). Due to recent reports on the various health-promoting effects of silk and other silkworm products, these products have been diversified to produce a range of value-added materials ranging from food ingredients to biomedical materials. This diversification has led to a growing interest in functional sericulture (Bae *et al*., 2016). Silkworm pupae, a byproduct of silk production, have been utilized as snacks or dietary supplements (Yun and Hwang, 2016). The high levels of unsaturated fatty acids and proteins in silkworm pupae have effects in improving both skin elasticity and muscle strength (Lee *et al*., 2019a). Recent studies have reported radical scavenging activities in two types of peptides isolated from silkworm pupae (Zhang *et al*., 2021); further studies have pursued the development of stable antioxidants using pupae-derived proteins and glucose complex (Attaribo *et al*., 2021).

An excessive accumulation of reactive oxygen species (ROS) accelerates fat oxidation, cell death, and inflammatory cytokine production, which may play a role in the development

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of cancer, arteriosclerosis, and other conditions related to aging (Browning and Horton, 2004). Both internal (e.g., mitochondria, inflammatory cell activation) and external (e.g., smoking, drugs, and environmental stressors) factors can increase ROS concentration within the body. The antioxidant supplements can help prevent age-related diseases by maintaining appropriate ROS levels in the body (Klaunig *et al*., 2011).

The current study compared the antioxidant effects of silkworm pupae according to sex, variety, and pupation time. The four color-based varieties such as Baegokjam (also known as White Jade), Goldensilk, Juhwangjam (also known as Orange silk), and YeonNokjam (also known as Pistachio silk) developed by the National Institute of Agricultural Sciences were used (Kang *et al*., 2011; Kim *et al*., 2020; Kweon *et al*., 2012; Lee *et al*., 1984). The samples classified as pupation day (7 to 11th day). In addition, antioxidant activities were compared across different extraction solvents to determine extraction conditions optimized for the highest retention of antioxidant content. The results of this study could be used for further development of food ingredients and functional materials using silkworm pupae.

Materials and Methods

Samples and Extraction

Silkworm powder (Uljin Silk Farm, Uljin, South Korea), mature silkworms (National Institute of Agricultural Sciences, Wanju, South Korea), and pupae samples (Uljin Silk Farm) were ground and stir-extracted in water and 30% ethanol for 24, 48, and 72 h. Samples of the different pupae varieties (Baegokjam, Goldensilk, Juhwangjam, and YeonNokjam; National Institute of Agricultural Sciences) were stir-extracted for 24 h using different solvents: water, various concentrations of ethanol (30, 50, 70, and 100%), and hexane. Next, the extracts were centrifuged at 10000 rpm for 10 min to isolate the supernatants. The isolated supernatants were then freeze-dried and stored in powder form; these were later re-dissolved in their respective extraction solvents for further analysis.

Analysis of Total Phenolic Content

The total phenolic content of the sericultural products (silkworm powder, mature silkworms, and pupae) were measured through the Folin-Ciocalteu colorimetric assay. Each sample extract (10 μL) was mixed with 200 μL of 2% Na_2CO_3 solution and stored at room temperature for 3 min. Subsequently, 10 μL of 50% Folin-Ciocalteu reagent (Sigma-Aldrich, St. Louis, MO, USA) was added to the mixture and stored at room temperature for 3 min. The total phenolic content was first determined using the absorbance at 750 nm and then converted to mg gallic acid equivalents (GAE)/g of samples based on the gallic acid value of the reference material (Yang *et al*., 2021).

Measurement of DPPH Radical Scavenging Activity

A radical reagent (DPPH) was used to measure antioxidant activity. The DPPH reagent (Sigma-Aldrich) was prepared as a 0.2 mM solution in ethanol. Aliquots of the sample extracts (10 μL) were transferred to a 96-well plate, after which 200 μL of the DPPH reagent was added and stored at room temperature for 10 min. The absorbance at 520 nm was measured using a spectrophotometer (Multiskan GO, Thermo Fisher, Waltham, MA, USA). A standard curve was then generated using Trolox, and the level of activity was calculated in mg Trolox equivalents (TE)/g of samples (Lee *et al*., 2021).

Measurement of ABTS Radical Scavenging Activity

To prepare the reagent for measuring antioxidant activity, a mixture of 7 mM ABTS (Sigma-Aldrich) and 2.5 mM potassium persulfate (Sigma-Aldrich) was allowed to react without exposure to light for 24 h. The resultant mixture was then diluted to attain an absorbance range of 1.4–1.5 at 735 nm. Next, 10 μL aliquots of the samples were transferred to a 96-well plate, after which 200 μL of the diluted ABTS solution was added and stored at room temperature for 10 min. The absorbance at 520 nm was measured using a spectrophotometer, and the results were quantified using the Trolox-based standard curve and converted to mg TE/g of samples (Lee *et al*., 2021).

Cell Culture and Measurement of Cytotoxicity

HepG2 cells were cultured on the Dulbecco's modified Eagle's medium (Caisson, North Logan, UT, USA) with 10% fetal bovine serum (Gendepot, Barker, TX, USA) and 1% penicillin/streptomycin (Caisson). To measure cytotoxicity, HepG2 cells were first treated with the samples at 500 μg/mL concentration for 24 h, and then treated with a 0.5 mg/mL 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reagent for 2 h. The resulting formazan crystals were dissolved in DMSO and absorbance at 570 nm was measured.

Measurement of Intracellular ROS Content

To determine intracellular antioxidant activity, HepG2 cells were transferred to a black 96-well plate (3×10^4 cells/well) and cultured for 24 h. The cells were treated with a 500 μg/mL solution of each sample and cultured for 24 h. Then, 25 μM 2', 7'-dichlorofluorescin diacetate (DCFA-DA) was added. After 1 h, the cells were treated with *tert*-butyl hydroperoxide (*t*-BHP) at a concentration of 1 mM for 1 h to induce oxidative stress responses. The intracellular ROS concentration was measured at 485/530 nm (excitation/emission); recorded data were expressed as values relative to those found in the control group (Lee *et al*., 2019b).

Statistical Analysis

The results were presented as average ± standard deviation. Analysis of variance was performed using the SAS Enterprise Guide 7.1 program (SAS Institute Inc., Cary, NC, USA), with the Duncan's multiple range test ($p<0.05$).

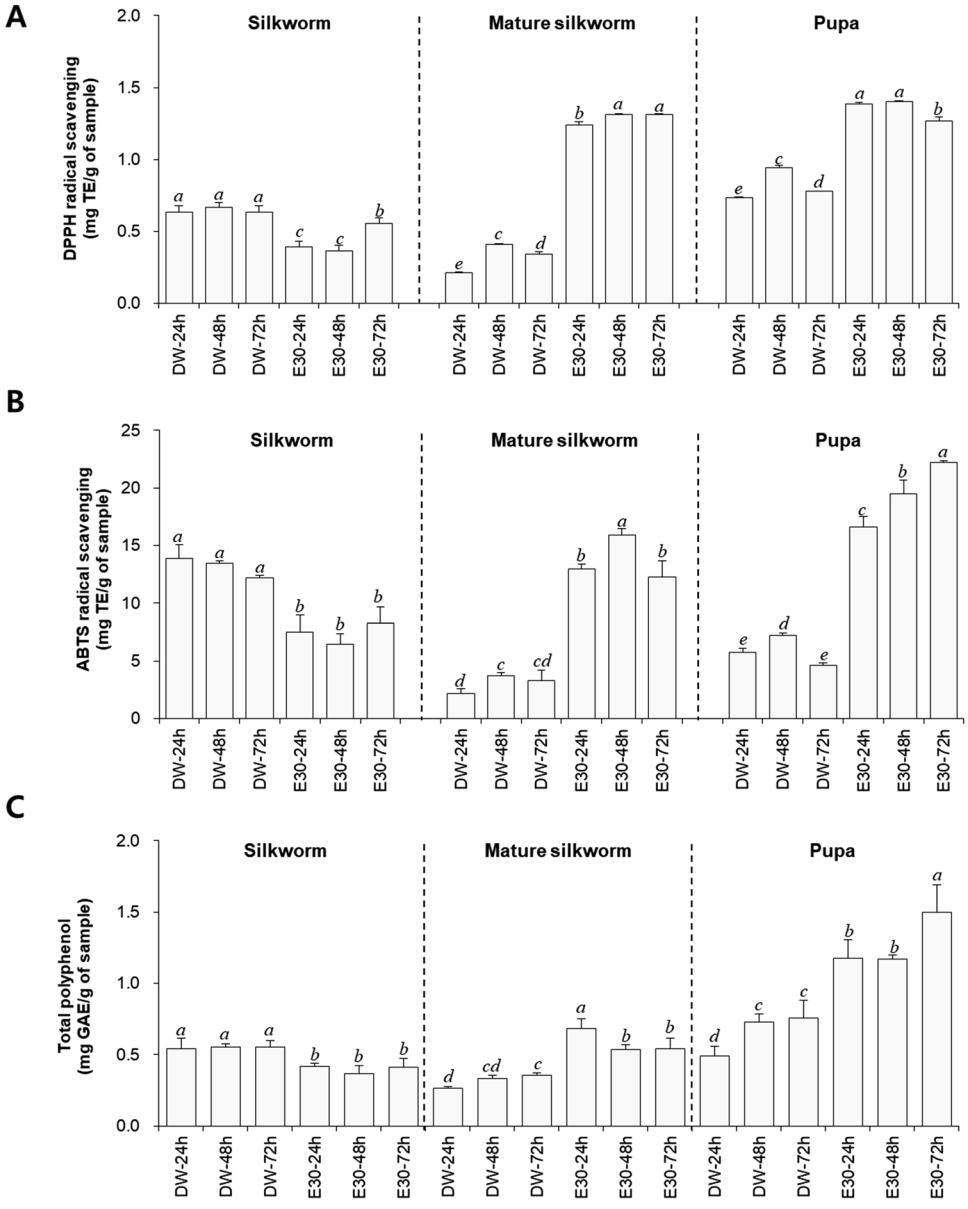

Fig. 1. Antioxidant properties and polyphenol contents of sericulture products. DPPH radical scavenging activity (A), ABTS radical scavenging activity (B), and polyphenol content of silkworm, mature silkworm, and pupa (C). The extracts were prepared using DW and 30% ethanol (E30) for 24, 48, and 72 hr. The data were mean±SD. Different letters are significant differences by Duncan's multiple range test within the same sericulture products ($p<0.05$).

Results and Discussion

Radical Scavenging Activity and Phenolic Content of Sericultural Products

The antioxidant effects and composition of sericultural products including silkworms, mature silkworms (Ji *et al*., 2017), and pupae were analyzed (Fig. 1). Silkworms showed higher levels of radical scavenging activity in water extracts than in 30% ethanol extracts. Meanwhile, radical scavenging activity levels for both mature silkworms and pupae were higher in 30% ethanol extracts than in water. DPPH radical scavenging activity was the highest in pupae samples at 1.4 mg TE/g (30% ethanol, 48 h extraction), and mature silkworms also showed similar levels of activity at 1.3 mg TE/g (48 h extraction) (Fig. 1A). ABTS radical scavenging activity was the highest in pupae samples at 22.3 mg TE/g (30% ethanol, 72 h extraction), while the activity for mature silkworm samples was highest at 15.9 mg TE/g (30% ethanol, 48 h extraction) (Fig. 1B). The amount of antioxidant phenolic compounds in pupae samples (30% ethanol) was the highest among the sericultural products studied at 1.2–1.5 mg GAE/g, with the values demonstrating variance based on extraction duration (Fig. 1C).

Phenolic compounds are antioxidants that directly scavenge free radicals. They remove hydrogen atoms from hydroxyl groups and donate them to radicals to form stable products, which curtail any further oxidation reactions (Shahidi and Ambigaipalan, 2015). While phenolic compounds are known to be secondary metabolites of plants, they are also found in the epidermis, wings, and digestive tracts of insects primarily due to phenolic compound accumulation from ingested plant tissues. Some phenolic compounds found in insects may also be synthesized through enzyme-mediated reactions, rather than through ingestion. The phenoloxidase enzyme, which is present in the epidermis, has been known to improve epidermal stability and strength through its contributions to the synthesis of phenolic compounds (Nino *et al*., 2021). This is based on the fact that higher phenolic contents in pupae may be attributable to their necessity for a more solid epidermis than that required by larvae. This, in turn, may be the basis for the observably higher levels of radical scavenging activity in pupae.

Radical Scavenging Activity of Silkworm Pupa Extract by Extraction Solvent

Antioxidant activity levels were compared across different extraction solvents using pupae, which showed the highest antioxidant activity among the sericultural products studied (Fig. 2). Extracts were prepared using water, ethanol (30, 50, 70, and 100%), or hexane as a solvent, and the radical scavenging activities of the samples were then compared. The levels of DPPH and ABTS radical scavenging activity were the highest in 30% ethanol extracts (4.14 and 19.3 mg TE/g of sample, respectively), while levels of antioxidant activity were generally lower in 100% ethanol and hexane extracts.

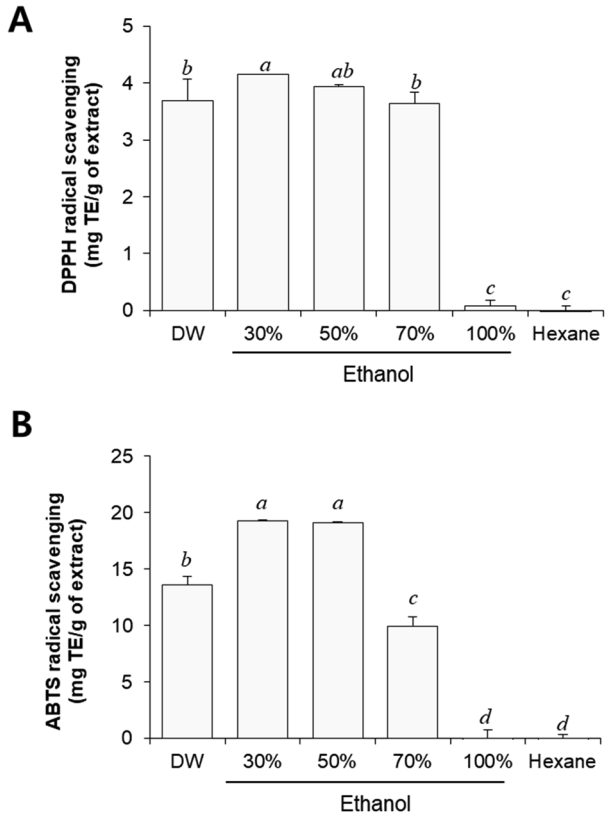

Fig. 2. Antioxidant properties of pupa according to extraction solvent. DPPH radical scavenging activity (A) and ABTS radical scavenging activity (B) were analyzed using DW, ethanol (30-100%) and hexane extracts. The data were mean±SD. Different letters are significant differences by Duncan's multiple range test within the same sericulture products ($p<0.05$).

In experiments using grain extracts such as oat or rice, where methanol, ethanol, ethyl acetate, or acetone were used as solvents, the retention of the extracted polyphenols tended to increase directly with solvent polarity. However, some tea-derived catechins demonstrated higher polyphenol contents in water extracts than those in 100% methanol or 100% ethanol extracts, suggesting that the optimal solvent could vary according to polyphenol type (Lee

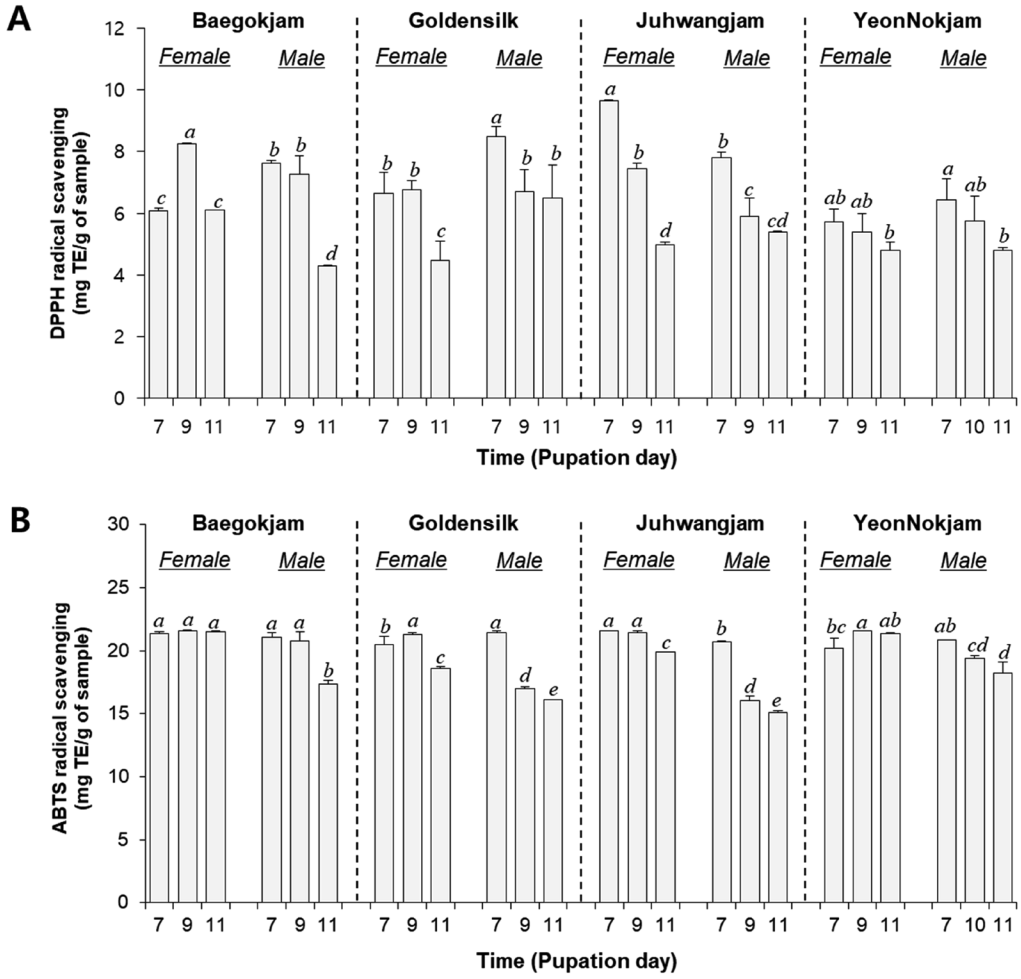

Fig. 3. Radical scavenging activity of pupa according to variety, pupation day, and sex. DPPH radical scavenging activity (A) and ABTS radical scavenging activity (B) were analyzed using four varieties (Baegokjam, Goldensilk, Juhwangjam, and YeonNokjam). The samples were separated according to pupation day (7 to 11th) and sex, than extracted using 30% ethanol. The data were mean±SD. Different letters are significant differences by Duncan's multiple range test within the same sericulture products (p<0.05).

et al., 2018). Therefore, an appropriate ratio of water and polar solvents is preferable over an absolute solvent. As the highest level of antioxidant activity among pupae extracts was observed in the 30% ethanol extract, subsequent experiments in this study were conducted using 30% ethanol extracts.

Radical Scavenging Activity by Silkworm Pupa Variety, Pupation Time, and Sex

To compare the antioxidant activity levels for different pupa varieties, the pupae of four color-based silkworm varieties (Baegokjam, Goldensilk, Juhwangjam, and YeonNokjam) were classified based on sex and pupation time before analysis. In the DPPH radical scavenging assay, pupae with shorter pupation days showed higher levels of activity; the only exception was the female Baegokjam, which showed the highest observable activity at 7 th pupation day (9.6 mg TE/g; p<0.05) (Fig. 3A). In the ABTS radical scavenging assay, short pupation days tended to correlate with higher levels of antioxidant activity, as demonstrated by male Baegokjam, which showed a 20% higher radical scavenging activity at 7th pupation day than at 11th pupation day (p<0.05). Additionally, levels of antioxidant activity were found to be higher in females than in males (Fig. 3B).

Color-based varieties of silkworms produce colored cocoons with varying compositions. Goldensilk, Juhwangjam, and YeonNokjam produce yellow, light pink, and pale green cocoons, respectively. This study aimed to compare the compositions of

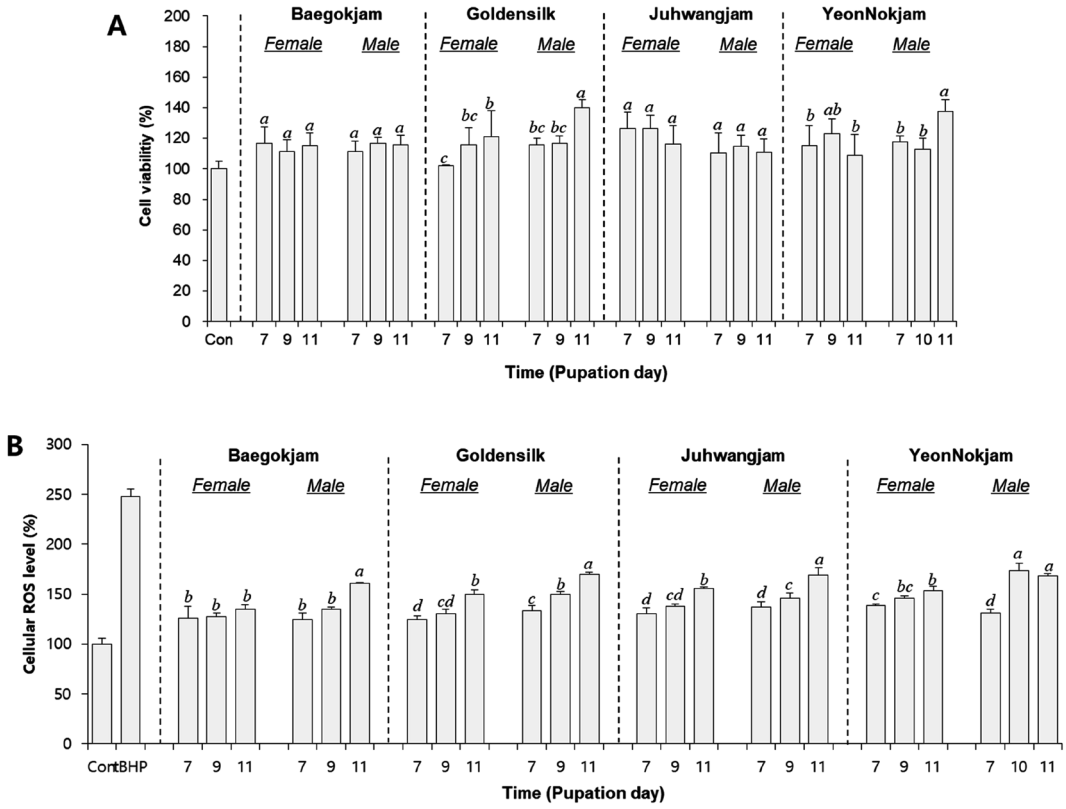

Fig. 4. Cellular ROS removal activity of pupa according to variety, pupation day, and sex. Cell viability in normal HepG2 (A) and ROS level in *t*-BHP treated HepG2 cells (B) were examined using four varieties (Baegokjam, Goldensilk, Juhwangjam, and YeonNokjam). The samples were separated according to pupation day (7 to 11th) and sex than extracted using 30% ethanol. The data were mean±SD. Different letters are significant differences by Duncan's multiple range test within the same sericulture products (p<0.05).

these color-based cocoon varieties; however, there was not a significant observable difference in the measured antioxidant activities among the silkworm varieties. This is likely due to the fact that no silk remains inside the pupae following cocoon production (Kang *et al*., 2011; Kim *et al*., 2020; Kweon *et al*., 2012; Lee *et al*., 1984). Yu *et al*. (2008) observed antioxidant activity in proteolytic pupae products. The antioxidant activities of proteins differ by the types and positions of amino acids, with methionine, histidine, tryptophan, and tyrosine showing the highest levels of antioxidant activity. As female pupae exhibit higher protein contents and male pupae have higher fat contents, female pupae are considerably more effective as antioxidants (Ryu *et al*., 2003).

Intracellular ROS Removal by Silkworm Pupa Variety, Pupation time, and Sex

A cytotoxicity test conducted prior to cell utilization did not show cytotoxicity upon sample treatment at 500 μg/mL (Fig. 4A). When the HepG2 cells were treated with *t*-BHP to test for ROS removal, a reduction in the ROS content (by 32–50%) was observed in the pupa extract treatment group (Fig. 4B). Pupae with shorter pupation days tended to be more effective at removing ROS, with male Baegokjam showing 23% higher ROS removal at 7th pupation day than at 11th pupation day (p<0.05). Shorter pupation days correlated with higher antioxidant effects and a greater ability to remove intracellular ROS, which is similar to the trend observed in experiments using DPPH and ABTS radicals.

ROS are sourced from external environments or are produced *in vivo* during metabolic processes. While the body has a defense system to maintain appropriate ROS concentrations, an excessive accumulation of ROS may trigger cell death, which in turn may lead to the development of geriatric conditions. Thus, the intake of antioxidants to help remove ROS may assist in the prevention

of the onset of cancer, cardiovascular diseases, and other metabolic and age-related conditions (Kim *et al*., 2012). In addition to high protein contents and the existence of phenolic compounds, silkworm pupae also have unsaturated fatty acids constituting over 70% of their total fat contents, which also seem to contribute to their antioxidant effects. Further studies on other functional pupa contents are warranted to investigate their influence on various biological activities on the basis of pupation time.

Summary

Among the sericultural products studied, silkworm pupae showed the most remarkable antioxidant effects that were attributable to their high phenolic contents (3.7 times higher than silkworms and 2.8 times higher than mature silkworms with 30% ethanol and 72 h extraction). When their radical scavenging activities were measured in different solvents, silkworm pupae samples demonstrated the highest levels of activity in 30% ethanol extracts (4.14 and 19.3 mg TE/g of extract for scavenging activities of DPPH and ABTS, respectively). While no large differences were observed between the different color-based silkworm varieties, specimens with shorter pupation times tended to have higher antioxidant effects. During the analyses of the radical scavenging activities associated with DPPH and ABTS, antioxidant activities were observably higher at 7th pupation day than at 11th pupation day (1.8 times higher for DPPH and 1.2 times higher for ABTS in male Baegokjam). In HepG2 cells with induced oxidative stress, ROS content was 0.77 times higher at 7th pupation day than at 11th pupation day (for male Baegokjam). The abundance of unsaturated fatty acids, peptides, and phenolic compounds in silkworm pupae contributes to ROS removal and other radical scavenging activities. Further research is needed to analyze pupae composition according to pupation time, sex, and varieties.

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