J. Soc. Cosmet. Sci. Korea
Vol. 45, No. 3, September 2019, 299-306

pISSN 1226-2587
eISSN 2288-9507
http://dx.doi.org/10.15230/SCSK.2019.45.3.299

진생베리 아미노산 복합체로부터 분리한 펩타이드의 항염, 주름개선 효과

강 상 문* · 박 청**,†

*에이앤펩(주) 기술연구소 · **대전대학교 생명과학부

(2019년 8월 30일 접수, 2019년 9월 23일 수정, 2019년 9월 27일 채택)

Anti-inflammatory and Wrinkle Improvement Effects of Peptides from Ginseng Berry Amino Acidic Complex

Sang Moon Kang* and Chung Park,†**

*R&D Center, ANPEP Inc. 13 Oksansandan 1-ro, Oksan-myeon, Heungdeok-gu, Cheongju-si, Chungcheongbuk-do, 28101, Korea
**Department of Life Science, Hyehwa Liberal Arts College, Daejeon University

(Received August 30, 2019; Revised September 23, 2019; Accepted September 27, 2019)

요 약: Ginseng berry(GB)는 진세노사이드Re를 함유하고 있으며, 항염, 피부 주름 완화의 기능을 가지고 있다. 본 연구에서는 Ginseng berry 아미노산 복합체의 TLC 분획 fraction 1, 2, 4를 확인하고 HPLC로 분석하였으며, fraction 1의 LC/MASS 분석을 통해 peptide (AP-1)를 동정하였다. AP-1에 의한 NO 생성 억제 효과를 조사하여 항염증 활성을 확인하였다. 또한 procollagen type I C-peptide (PIP) ELISA kit를 이용한 collagen 합성은 대조군 대비 50% 이상의 효과를 보였다. 이상의 결과로부터 진생베리 아미노산 복합체로부터 분리한 펩타이드는 항염과 주름개선 효능을 가진다고 사료되며, 향후 항염 및 항노화 화장품 원료로서의 이용 가능성을 보였다.

Abstract: Ginseng berry (GB) contains Ginsenoside Re and has anti-inflammatory and anti-wrinkle properties. In this study, TLC fractions 1, 2, and 4 of the ginseng berry amino acid complex were identified and analyzed by HPLC. And identified a peptide (AP-1) by LC/MASS analysis of fraction 1. The anti-inflammatory activity was confirmed by investigating the inhibitory effect of AP-1 on NO production. In addition, collagen synthesis using procollagen type I C-peptide (PIP) ELISA kit was 50% higher effective than that of the control group. From these results, the peptide isolated from ginseng berry amino acid complex is considered to have anti-inflammatory and anti-wrinkle effect, and may be useful as an anti-inflammatory and anti-aging cosmetic raw material.

Keywords: *Ginseng berry, peptide, anti-inflammatory, wrinkle improvement, anti-aging*

1. Introduction

Panax ginseng is particularly common and widely used in oriental countries, because of its property of boosting the immune system, as well as providing vigor and enhancing sexual activity[1,2]. It contains medicinal ingredients, including saponin, polysaccharide, polyacetylene, phenols, gomisin, acidic peptide and carbohydrate[3-5]. The major active components are ginsenosides, a class of steroid glycosides and triterpene saponins naturally occurring in the root, leaf and berry[6,7] Studies of the pharmacological roles of ginsenosides have focused mostly on their anticancer, antioxidative, and immunostimulatory activities. A number of recent studies, however, have presented evidence showing that ginsenosides

† 주 저자 (e-mail: pchung@dju.kr
call: 042-280-4809)

could be used to prevent and treat a variety of inflammatory diseases via anti-inflammatory functions[8]. Ginsenosides are divided into three major groups based on the triterpene aglycones: panaxadiol, panaxytriol, and olenolic acid derivatives[9]. In addition to amino acids, nucleic acids, various enzymes and inorganic compounds are obtained from ginseng[10].

The ginseng berry has various bioactivities, including antidiabetic, anticancer, anti-inflammatory, and anti-oxidative properties. A recent study reported that the ginseng leaf and berry have higher levels of certain ginsenosides than the ginseng root, and that the active anti-aging component of the ginseng berry, syringaresinol, has the ability to stimulate longevity via gene activation[11-13]. Currently, ginseng berry extract is being evaluated in clinical and preclinical trials because it has high levels of active compounds, especially ginsenoside Re and vitamin E[6,14]. Despite the many known beneficial effects of ginseng, peptide components of ginseng berry on skin aging are poorly understood.

The proteins that make up the skin include collagen and elastin. Collagen synthesis and decomposition process is related to the wrinkles of the skin. If this process is abnormally imbalanced, wrinkles may increase and cause skin aging. Several studies have shown that collagen peptides or supplements containing collagen may help slow the aging of skin by reducing wrinkles and dryness[15-17].

In this study, we isolate amino acidic complex from ginseng berry and to evaluate skin benefit of the anti-inflammatory and wrinkle improvement.

2. Materials and Methods

2.1. Chemical Analysis and Reagents

High-performance liquid chromatography (HPLC, HA Shimadzu, LC-20AD, Japan) system with a Shim-pack VP-ODS C18 column (250 mm × 4.6 mm, 5 μm) was used. All solvents used were of analytical grade. ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded on FT-NMR system (JNM-ECX 400, JEOL, Japan) and FT-NMR system, (AVANCE Ⅲ 500, Bruker, Germany) instruments with chemical shift (δ) data. Merck silica gel (0.063 - 0.2 mm)was used for normal phased column chromatography. Silica gel 60 F_{254} coated on aluminum plates by Merck were used for thin layer chromatography (TLC). Ginseng berry (GB) was harvested in Yeongju-si, Gyeongsangbuk-do, Korea, from 2017 to 2018.

2.2. Preparation of Ginseng berry Amino Acidic Complex

The whole ginseng berry amino acidic complex was thoroughly washed with water, dried and grind into powder. The dried powder (100 g) was extracted with 70% ethanol (2 L) for 72 h, and the extract was incrassated by a rotary evaporator for 3 h. To remove the ethanol from the extract, it was mixed with water and incrassated again. Subsequently, the extracted liquid was filtered through filter paper and frozen on a freezing tray for 48 h. This material was suspended in water and fractionated for each solvent. Each solvent was separated into n-hexane, dichloromethane, ethylacetate (EtOAc), n-butanol, and water sequentially. Each run was performed twice. Each fraction was concentrated using a rotary concentrator. Separation was performed by polarity through the fraction. The fractions were further separated by checking the peptide content through protein quantification.

2.3. Components Analysis by TLC and HPLC

Ginseng berry extract solution was used for TLC and HPLC analysis. The developing solvent used in the TLC analysis was found to be most ideally separated under the conditions of Methylene chloride: Methanol, and proceeded sequentially from Methylene chloride: Methanol to DW 100%. and 1 L for each condition was developed to confirm the fraction. After scraping the band identified as a single substance separated by TLC, extracted with 70% ethanol extraction, filtration, vacuum drying process, and then dissolved in 100% ethanol and filtered using a syringe filter (Milipore 0.45 μm). The filtrate was used for protein quantitation, HPLC, LC/ESI-MS/MS analysis. HPLC analysis was performed by gradient elution using 0.1% trifloacetic acid and acetonitrile containing 0.1% trifloacetic acid, and HPLC separation conditions are shown in Table 1.

Table 1. HPLC Condition for Separation of EtOAc Fractions of Ginseng Berry Amino Acidic Complex

Condition of HPLC analysis				
Column	Shim-pack VP-ODS C18 column (L : 250 mm, LD : 4.6 mm, 5 μm)			
Detector	UVD 170s DIONEX			
Detection wavelength	216, 254 nm			
Flow rate	1.0 mL/min			
Injection volume	50 μL			
Mobile phase conditions for HPLC gradient-elution	Program order	Time (min)	0.1% TFA[1] in D.W (%)	0.1% TFA[1] in 50% ACN[2] (%)
	1	0	100	0
	2	10	75	25
	3	20	50	50
	4	30	25	75
	5	40	0	100
	6	45	100	0

[1] TFA : trifloacetic acid, [2] ACN : acetonitrile

2.4. Component Analysis using LC/ESI–MS/MS

The LC instrument is a Thermo-Finnigan surveyor instrument (EN 61000-4-11, Thermo scientific, USA) (column spec. U-VD Spher Pur C18-E 1.8um, 50 x 2.0mm Cat-No.N0520E181UVC), autosampler, PDA-UV detector. Mass spectrometric analysis device was used as Mass spectrometer (JEOL JMS-700, JEOL, Japan). The injection volume is 5 μL, 200 μL/min of flow rate, and the developing solvent condition is 75% of 0.1% formic acid (in DW, solvent A) and 25% of 0.1% formic acid (in acetonitrile, solvent B).

2.5. Cell Culture and Reagents

The RAW264.7, mouse macrophage cell line was maintained in dulbecco's modified eagle's aedium (DMEM, Gibco, USA), containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (Invitrogen, USA), at 37 ℃, under 5% CO_2.

The human keratinocyte cell lines (HaCaT cell) was maintained in DMEM (Gibco, USA), containing 10% FBS (Gibco, USA) and 1% penicillin/streptomycin, at 37 ℃, under 5% CO_2.

2.6. Cell Viability Assay

Cell viability was measured using the MTT (USB Corp., USA) assay. The cells (HaCaT cell, Human fibroblast) were treated with 50, 500, and 5000 μg/mL ginseng berry for 24 h. Then, MTT reagent (1 mg/mL) was added to each well, and the cells were incubated for 3 h. The medium was removed, and the cells were solubilized with DMSO. The absorbance was measured by spectrophotometer at a wavelength of 570 nm.

2.7. Nitric Oxide Determination

The concentration of NO in the culture supernatants was determined as nitrite, a major stable product of NO. The cells were incubated in the presence or absence of 1, 2.5, 5.0 and 10 μg/mL ginseng berry amino acidic complex for 1 h and induced by LPS (200 ng/mL) for 24 h. 50 mL of cell culture supernatants were incubated with equal volume of Griess reagent for 30 min at room temperature. The absorbance was measured by a spectrometer at a wavelength of 540 nm.

2.8. Collagen Synthesis

Collagen biosynthesis was measured using procollagen type I C-peptide (PIP) ELISA kit. Using human fibroblasts cultured in DMEM, 2×10^5 cells were dispensed into 24 wells, and after 24 h, fractions were added with experimental concentration, exchanged with serum-free medium for 24 h, and cell cultures were collected. Synthesized collagen was measured using a PIP collagen assay kit. The 20 μL of the cell culture solution was

put into a 96 well plate uniformly coated with primary collagen antibody, and the antigen-antibody reaction was light-blocked in a 37 ℃ incubator for 3 h, washed four times with PBS, and then the chromophore was bound. After the secondary collagen antibody was added to the kit and reacted again for 15 min, color development-causing substance was added to induce color development at room temperature for 15 min, and then the color development was stopped by adding 1 M sulfuric acid and absorbed at 450 nm by ELISA reader Measure.

2.9. Statistical Analysis

All data are expressed as means ± standard deviations. Differences between the control and the treatment group were evaluated by one way ANOVA. $P < 0.05$ or 0.01 was considered statistically significant.

3. Results

3.1. Ginseng Berry Amino Acidic Complex Isolation by Silica Column

In order to analyze the fractions more precisely, the fractions were concentrated and further purified by column. The silica column was used for purification and the mobile phase was found to be most ideally separated under the conditions of CH_2Cl_2: MeOH = 2 : 1.1 L for each condition was checked by TLC for each fraction obtained after the column was run to confirm the material purification conditions through the column.

When separated and purified through silica, the substance identified as a single substance in fraction 1 and 2 was identified on TLC. Protein quantification was to confirm that

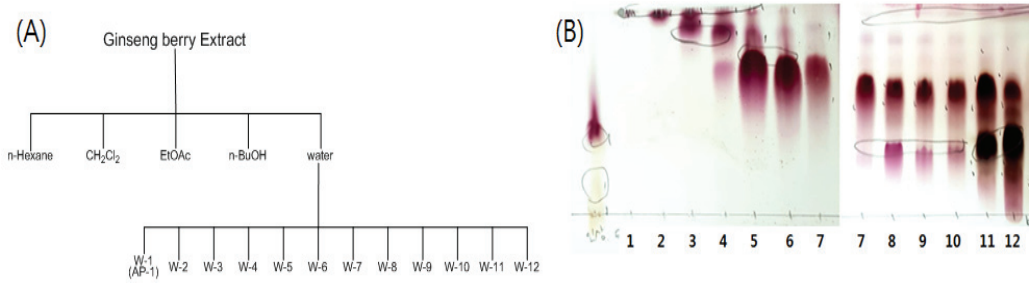

Figure 1. Fraction of ginseng berry amino acidic complex. (A) Diagram for the fractionation of mixture extracted from ginseng berry, and (B) TLC chromatograms of effluent fraction from silica gel column chromatography.

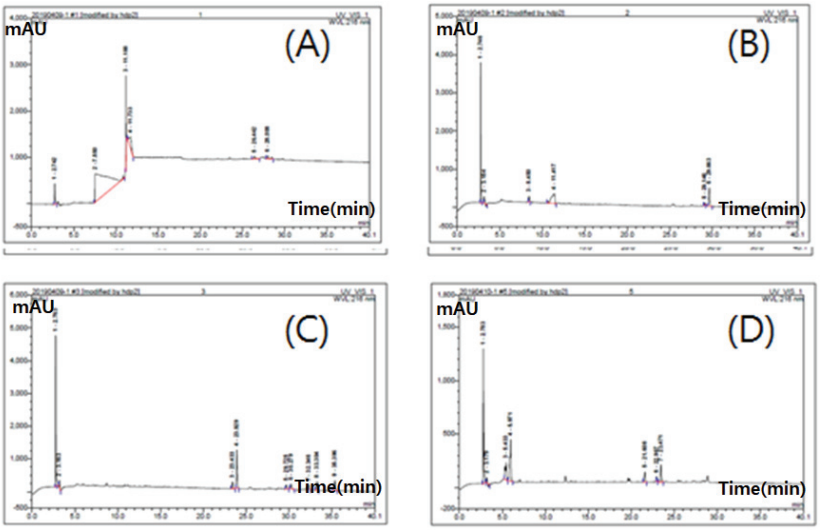

Figure 2. HPLC chart of ginseng berry fraction. (A) ginseng berry fraction 1, (B) ginseng berry fraction 2, (C) ginseng berry fraction 3 and (D) ginseng berry fraction 4. Ginseng berry extract was identified by fractionation of the open column using HPLC.

the substance is a protein, and when the silica column was run through the same length and the same solvent in order to confirm the reproducibility of the material, the reproducibility was also confirmed by obtaining the same result (Figure 1).

Protein quantification experiments confirmed that the protein contained 80% ~ 90% of fractions. A single peak was confirmed in fractions 1, 2, and 4, by HPLC, and the substance was further confirmed through NMR and Mass (Figure 2).

3.2. LC/ESI-MS/MS and NMR Analysis of Ginseng Berry Amino Acidic Complex

In order to confirm the molecular weight identified through HPLC, the molecular weight was confirmed through Mass and confirm the substance by NMR. In order to confirm the structure of the isolated peptide, the substance was confirmed through NMR. In the structure analysis of fraction 1, it was confirmed that the peak of S - CH_3 was shifted by the influence of sulfur at δ 1. 3, and many CH_2 and CH_3 peaks were also confirmed. In addition, four peaks of COOH or CONH were confirmed through ^{13}C-NMR. When confirmed by the protein quantitative value, it was confirmed that the protein content of the fraction of which purity was confirmed by HPLC was over 80%, and other complex peptide compounds containing Met (AP-1) were identified.

3.3. Ginseng Berry Amino Acidic Complex Inhibits NO Production in LPS-induced RAW264.7 Cells

NO is synthesized from L-arginine in a reaction catalyzed by the nitric oxide synthase (NOS) family of proteins. iNOS is primarily responsible for the production of NO in inflammatory processes, is not typically expressed in resting cells, but is induced by certain cytokines or microbial products. The aberrant release of NO can lead to the amplification of inflammation, as well as tissue injury. In this study, we investigated the effect of ginseng berry fraction hydrolysate on NO production in RAW264.7 cells. To examine the effect of ginseng berry hydrolysate on NO production, we measured the level of nitrite using the Griess reaction.

As shown in Figure 4, ginseng berry fraction 1 (W-1) (1, 2.5, 5.0 and 10 μg/mL) significantly inhibited the LPS-induced nitrite production in a concentration dependent manner. However, cytotoxicity was not observed in ginseng berry protein hydrolysate treated RAW264.7 cells at the concentration range of 1 ~ 10 μg/mL. Also MTT assay was carried out using HaCaT cells to confirm fibroblast toxicity of ginseng berry fraction 1. Toxicity was not observed in HaCaT cells, and at high concentration of 5000 μg/mL were found to stimulate cell growth.

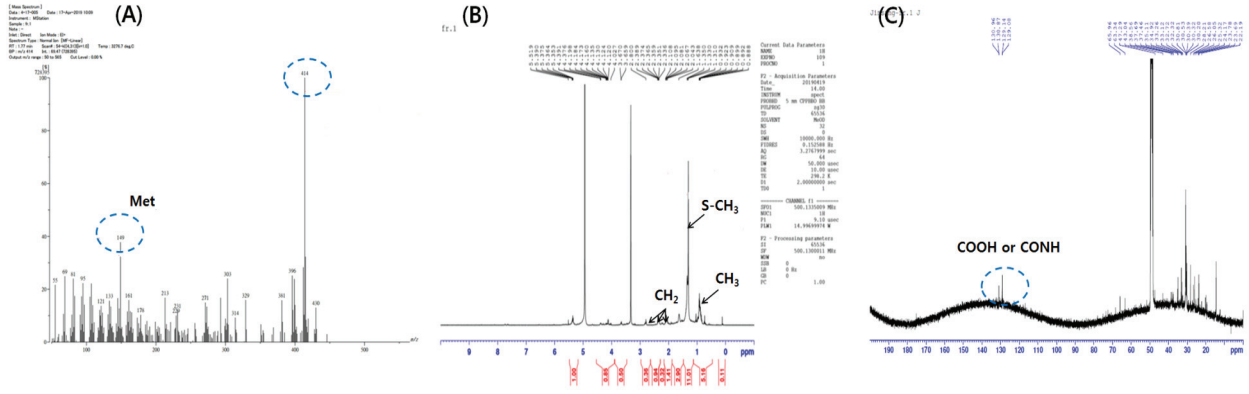

Figure 3. LC/ESI-MS/MS (A) ^{1}H-NMR analysis, (B) ^{13}C-NMR analysis and (C) ginseng berry amino acidic complex.

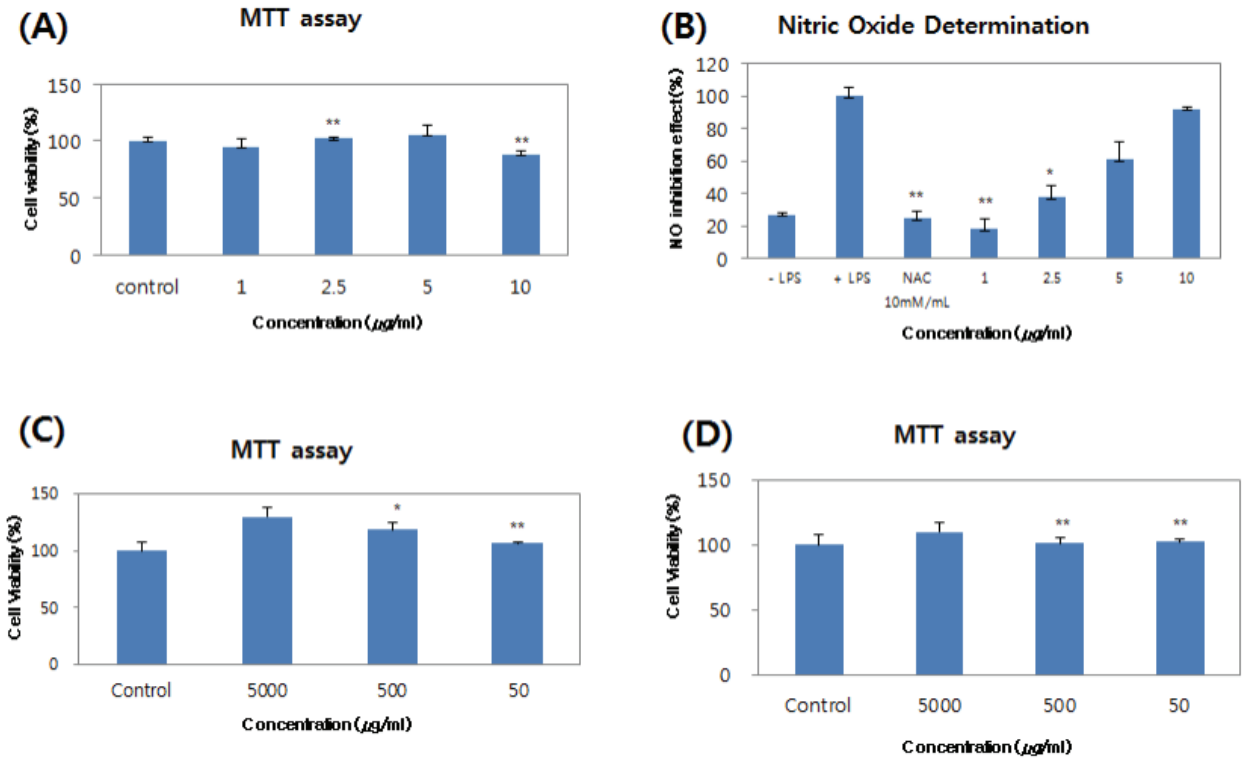

Figure 4. Effects of ginseng berry fraction 1(W-1) on NO production in LPS-induced RAW264.7 cells. (A) Raw264.7 cell viability was measured by MTT assay, (B) The cells were pretreated with the indicated concentrations of ginseng berry for 1 h and then further incubated with LPS (200 ng/mL) for 24 h and (C) HaCaT cell viability was measured by MTT assay. (D) Human fibroblast cell viability was measured by MTT assay. The results are mean ± SD (N = 3, $^{*}p < 0.01$ *vs.* LPS-untreated control. $^{**}p < 0.01$ *vs.* LPS-treated control).

3.4. Collagen Synthesis of Ginseng Berry Amino Acidic Complex

The fraction 1 and fraction 4 containing single peptide in ginseng berry fractions increased collagen synthesis. We determined the effect of ginseng berry fractions on the synthesis and secretion of procollagen Type I C-peptide. As shown in Figure 5, fractions 1 (AP-1, 20 mg/mL) increased collagen biosynthesis and PIP value of ginseng berry extract, control and AP-1 was 380, 321 and 520 ng/mL respectively. AP-1 showed 43.6% higher than the ginseng berry extract and 61.9% higher than the control.

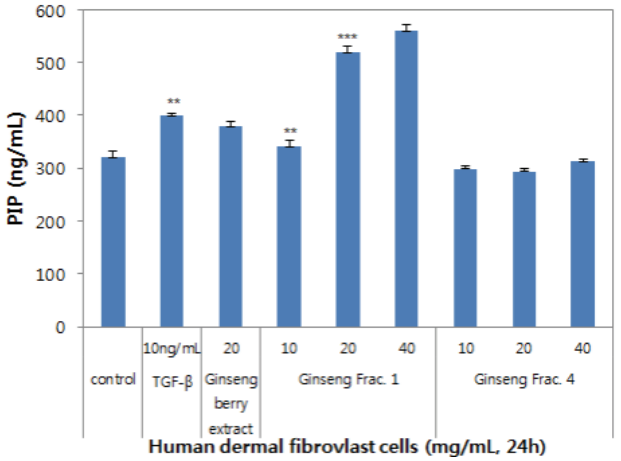

Figure 5. Collagen synthesis activity assay of ginseng berry (Ginseng Frac. 1 = Ginseng berry fraction 1, W-1, AP-1, Ginseng Frac. 4 = Ginseng berry fraction 4, W-4). The results are mean ± SD (N = 3).

4. Discussion and Conclusion

Ginseng berry extract was diluted and suspended in water for polar fractionation in order to separate. As solvents n-hexane, dichloromethane, ethylacetate and n-butanol were used, and fractionation was performed twice for 12 h each. After confirming the substance by TLC for each fraction, the peptide content was confirmed through protein quantification to further separate and identify the water layer showing the most peptide content. After confirming the conditions of resolution of each fraction, the secondary separation was performed through the open column. Before proceeding with the separation condition, the CH_2Cl_2 : MeOH = 2 : 1 condition showed the most ideal resolution. The open column changed the solvent from CH_2Cl_2 to CH_2Cl_2 : MeOH sequentially from 100 : 0, 80 : 20, 60 : 40, 40 : 60, 20 : 80 to isolate peptide compounds (W-1, AP-1). Nitric oxide synthase activity assay and collagen synthesis activity assay were performed to confirm anti-inflammatory effect and anti-aging effects with peptide compounds (W-1, AP-1). When the peptide mixture (AP-1) was identified by the nitric oxide synthase activity assay, it showed good efficacy by concentration, and collagen synthesis also showed 61.9% better efficacy. From the previous studies, AP-1 showed about 43.6% better collagen synthesis than ginseng berry extract(Figure 5). The functional peptide derived from ginseng berry developed through this study is expected to have various applications, which are declining while attracting the researchers' attention to the functionality of peptides other than ginsenosides or polysaccharides of existing ginseng. The researchers found that ginseng berry contained the highest protein and peptide among ginseng and ginseng by-products, and that bioconversion changed the pattern of proteins and peptides. These results suggested that ginseng berry peptide has potential as a antiaging cosmetic ingredient.

Reference

1. K. Y. Nam, Clinical application and efficacy of Korean ginseng, *J Ginseng Res*, **26**(3), 111 (2002).
2. J. H. Kim, All about Korean ginseng, Korean Society of Ginseng, 80 (2008)
3. J. N. Hu, J. H. Lee, J. A. Shin, J. E. Choi, and K. T. Lee, Determination of Ginsenosides content in Korean ginseng seeds and roots by high performance liquid chromatography, *Food Sci. Biotechnol.*, **17**(2), 430 (2008).
4. B. R. Yoon, Y. J. Lee, H. D. Hong, and Y. C. Lee and Y. C. Kim and Y. K. Rhee, K and T. Kim, and O. H. Lee, Inhibitory effects of *Panax ginseng* C. A. Mayer treated with high temperature and high pressure on oxidative stress, *Korean J. Food & Nutr.*, **25**(4), 800 (2012).
5. S. K. Ko, O. S. Cho, H. M. Bae, B. W. Yang, B. O. Im, Y. T. Hahm, K. N. Kim, S. H. Cho, J. Y. Kim, S. H. Chung, and B. Y. Lee. Changes in ginsenoside composition of white ginseng by fermentation, *Food Sci. Biotechnol.*, **18**(1), 253 (2009).
6. C. Z. Wang, B. Zhang, W. X. Song, A. Wang, M. Ni, X. Luo, H. H. Aung, J. T. Xie, R. Tong, T. C. He, and C. S. Yuan. Steamed American ginseng berry: ginsenoside analysis and anticancer activities, *J. Agric. Food Chem.*, **54**(26), 9936 (2006).
7. J. Kim, S. Y. Cho, S. H. Kim, S. Kim, C. W. Park, H. W. Park, D. B. Seo, and S, S. Shin, Ginseng berry, a promising anti-aging strategy: recent opinions on the biological effects of a traditional Korean ingredient. *J Adv Res Biotech.*, **1**(2), 8 (2016).
8. J. H. Kim, Y. S. Yi, M. Y. Kim. and J. Y. Cho, Role of ginsenosides, the main active components of Panax ginseng, in inflammatory responses and diseases, *J Ginseng Res*, **41**(4), 435 (2017).
9. Z. Xi, W. Chen, Z. Wu, Y. Wang, P. Zeng, G. Zhao, X. Li, and L. Sun, Anti-complementary activity of flavonoids from Gnaphalium affine D. Don, *Food Chem*, **130**(1), 165 (2012).
10. M. Morimoto, S. Kumeda, and K. Komai, Insect antifeedant flavonoids from Gnaphalium affine D. Don, *J. Agric. Food Chem.*, **48**(5), 1888 (2000).
11. A. S. Attele, J. A. Wu, and C. S. Yuan. Ginseng pharmacology: multiple constituents and multiple, *Biochem. Pharmacol.* **58**(11), 1685 (1999).

12. S. K. Ko, H. M. Bae, O. S. Cho, B. O. Im, S. H. Chung, and B. Y. Lee, Analysis of ginsenoside composition of ginseng berry and seed, *Food Sci. Biotechnol.*, **17**(6), 1379 (2008).
13. E. J. Kwon, S. Hong, M. M. Kim, J. W. Kim, D. W. Kim, and K. T. Chung, Effects of ginseng berry water extract on the polysaccharide hydrolysis of extracellular enzymes and intracellular PTP1B and AKT1, *J. Life Sci.*, **24**(9), 1006 (2014).
14. K. J. Choi and D. H. Kim, The characteristics and antioxidant activity of non-enzymatic browning products from fresh ginseng extracts and those with arginine or glucose, *J Ginseng Res*, **5**(1), 8 (1981).
15. P. Brenneisen, H. Sies, and K. Scharffetter-Kochanek, Ultraviolet-B irradiation and matrix metalloproteinases: from induction via signaling to initial events, *Ann. N. Y. Acad. Sci.*. **973**, 31 (2002).
16. L. Baumann, Cosmetic dermatology, principles and practice, 10, McGraw-Hill Co., New York (2002).
17. M. K. Lee, D. H. Kim, T. S. Park, and J. H. Son, Antioxidant and anti-wrinkling effects of extracts from *Vitex trifolia* L. *J. Appl. Biol. Chemi.,* **58**(2), 125 (2015).