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

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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, antioxidant, and immunostimulatory activities. A number of recent studies, however, have presented evidence showing that ginsenosides
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. 1H and 13C 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 F254 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% trifluoacetic acid
and acetonitrile containing 0.1% trifluoacetic acid, and HPLC
separation conditions are shown in Table 1.
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 °C, under 5% CO₂.

The human keratinocyte cell lines (HaCaT cell) was maintained in DMEM (Gibco, USA), containing 10% FBS (Gibco, USA) and 1% penicillin/streptomycin (Invitrogen, USA), at 37 °C, under 5% CO₂.

2.6. Cell Viability Assay

Cell viability was measured using the MITT (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, MITT 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 × 10⁵ cells were dispersed 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 treated with 50, 500, and 5000 μg/mL ginseng berry for 24 h.

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

<table>
<thead>
<tr>
<th>Condition of HPLC analysis</th>
<th>Column</th>
<th>Shim-pack VP-ODS C18 column (L : 250 mm, LD : 4.6 mm, 5 μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detector</td>
<td>UVD 170s DIONEX</td>
<td></td>
</tr>
<tr>
<td>Detection wavelength</td>
<td>216, 254 nm</td>
<td></td>
</tr>
<tr>
<td>Flow rate</td>
<td>1.0 mL/min</td>
<td></td>
</tr>
<tr>
<td>Injection volume</td>
<td>50 μL</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Program order</th>
<th>Time (min)</th>
<th>0.1% TFA¹ in D.W (%)</th>
<th>0.1% TFA¹ in 50% ACN² (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>75</td>
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<td>0</td>
<td>100</td>
</tr>
<tr>
<td>6</td>
<td>45</td>
<td>100</td>
<td>0</td>
</tr>
</tbody>
</table>

¹ TFA : trifloacetic acid, ² ACN : acetonitrile

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put into a 96 well plate uniformly coated with primary collagen antibody, and the antigen-antibody reaction was light-blocked in a 37 °C 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 CH₂Cl₂: 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 - CH₃ was shifted by the influence of sulfur at δ 1.3, and many CH₂ and CH₃ peaks were also confirmed. In addition, four peaks of COOH or CONH were confirmed through ¹³C-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) ¹H-NMR analysis, (B) ¹³C-NMR analysis and (C) ginseng berry amino acidic complex.
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 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).

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$_2$Cl$_2$ : MeOH = 2 : 1 condition showed the most ideal resolution. The open column changed the solvent from CH$_2$Cl$_2$ to CH$_2$Cl$_2$ : 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