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연구논문

Peptide Hydrolysates from *Astragalus membranaceus* Bunge Inhibit the Expression of Matrix Metalloproteinases in Human Dermal Fibroblasts

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Abstract: Inhibition effects of peptide hydrolysates from *Astragalus membranaceus* Bunge. on the expression of the matrix metalloproteinases (MMPs) in human dermal fibroblasts were evaluated *in vitro*. Crude peptides were obtained by the hydrolysis of proteins extracted from *A. membranaceus*. Peptides were purified partially by the basis on the molecular weight using 40% polyacrylamide gel electrophoresis before treatment with human dermal fibroblasts. Basis on the dose-effect experiments, expressions of MMPs including MMP-1, MMP-3, MMP-8, MMP-13 in human dermal fibroblasts were evaluated. Expressions of MMP-1, MMP-3, MMP-8 and MMP-13 were reduced in 43%, 5%, 22% and 57% respectively. The mass spectrometric analysis of partially purified peptides from *A. membranaceus*, which strongly inhibit expressions of MMPs, indicated that the peptides were composed of molecules below 1500 Da.

Keywords: *Astragalus membranaceus*, Peptide hydrolysate, Matrix metalloproteinase, Human dermal fibroblast

1. INTRODUCTION

Collagen is one of the major compounds in connective tissues of mammals. In humans, collagen comprises approximately 40% of the protein in the body, providing firmness, suppleness, constant renewal and elasticity of skin cells. Skin aging expressed as winkles is the final result of imbalance of synthesis-degradation of collagen. In this process, the synthesis of collagen decreases and its degradation increases. The degradation occurs via matrix metalloproteinase, a large family of calcium-dependent zinc-containing endo-peptidases, especially MMP-1, MMP-3, MMP-8, and MMP-13 [1]. There are many active ingredients that inhibit MMPs and collagen degradation and stimulate collagen synthesis, were used in cosmetic formulas. Such as active ingredients derived from plant peptide hydrolysates have gained popularity owing to their good effects and because they are non-toxic at the recommended dosages.

The *A. membranaceus* root, which is one of the most popular health-promoting plants in East Asia, has been used to treat the common cold, diarrhea, fatigue, anorexia and cardiac diseases for more than 2000 years [2]. More than 100 compounds exist in root, including triterpenoid saponins and flavonoids [3-9]; however, few studies of the peptide hydrolysate of *A. membranaceus* have been conducted to date. In this study, we evaluated the effects of inhibition of *A. membranaceus* peptides on the expression of MMPs in human dermal fibroblasts.

2. MATERIALS AND METHODS

2.1. Materials

A. membranaceus roots were purchased from Korean market. Desalting Protein/peptide Toptip™ - TT3CAR was supplied by Glygen corp. (USA). Dulbecco's modified eagle media (DMEM) and Immobiline DryStrips (pH 3-10 linear, 24 cm long) were supplied by GE Healthcare (USA). Dulbecco's phosphate buffer saline (DPBS) and fetal bovine serum (FBS) were supplied by WelGENE Inc. (Korea). Human dermal fibroblasts were supplied by Ajou Cosmetic Science lab. Chemicals of electrophoresis were purchased from Aldrich Sigma (USA)

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and GE Healthcare (USA).

2.2. *A. membranaceus* peptides (AMPs) preparation

A. membranaceus proteins were digested by combination with trypsin and chymotrypsin (10:1, w/w). Briefly, enzymes were dissolved in 10 mM $CaCl_2$ and 2 mM HCl to a stock concentration of 20 ×. For digestion, the substrate - enzyme ratio was 25:1 (w/w). The hydrolyzation process was conducted in 50 mM ammonium bicarbonate solution at pH 8~9 for 12~18 hours. The reaction was terminated by heating the samples to 80°C for 5 minutes, after which the hydrolysated suspension was centrifuged at 10,000 rpm for 10 minutes. The supernatant was used as the AMPs solution.

2.3. Separation of AMPs

AMPs were separated based on molecular weight by electrophoresis on a 40% polyacrylamide (acrylamide: bisacrylamide, 19:1, w/w) gel. Briefly, the peptides mix was loaded onto the gel with agar stacking gel solution. The electrophoresis process was subjected 10 mA/gel for 1 hour and then 40 mA/gel until tracking line crossed half of the gel, at which time the gels were divided into 10 parts following the dimension from the top to bottom of gels (from PEP1 to PEP10, each portion was 15 mm long). The peptides in each portion were extracted in 50% acetonitrile (ACN) supplemented 1% acetic acid. Next, the extracts were evaporated under nitrogen to give higher concentrations. Peptides extracts were desalted using protein/peptide Toptips™ - TT3CAR that had been activated in 50% ACN solution added 0.1% formic acid before use. Contaminating salts were eliminated by the addition of 0.1% formic acid and peptides were eluted with 50% ACN solution. The elutions were evaporated under vacuum, after which the residual portions were dissolved in DMEM for testing against to human dermal fibroblasts.

2.4. Human dermal fibroblast cell culture

Human dermal fibroblasts were seeded at 5×10^5 cells/plate in 10 mL media (DMEM + 10% fetal bovine serum (FBS) supplemented 1% antibiotic antimycotic (penicillin 10,000 unit/mL, streptomycin 10,000 μg/mL, amphotericin B 25 μg/mL). The cells were incubated at 37°C under 5% CO_2, 95% air for 3 days. The separated peptides were added into the culture media at 100 ppm. Additionally, cells were incubated in media without peptides as the control. After 3 days cultivation, human dermal fibroblasts were harvested by scraping and then washed in DPBS of diluted 1/10 concentration. Proteins in human dermal fibroblasts were extracted in lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.002% bromophenol blue, 2% carrier ampholyte, 4% protease inhibitor cocktail) for 5 hours, then centrifuged at 13,500 rpm for 10 minutes. The supernatants were subsequently quantified by Bradford assay in an ELISA microreader at 595 nm using bovine albumin as the standard.

2.5. 2-D PAGE (polyacrylamide gel electrophoresis)

Isoelectric focusing (IEF): The extracted proteins were loaded onto immobiline DryStrips (pH 3-10 linear, 24-cm-long) that had been rehydrated for 12 hours in 600 μL of rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% carrier ampholyte, 10% glycerol, 0.002% bromophenol blue). Each strip was loaded with 100μg proteins, 2% DNAse, and 5% protein marker. Proteins were isoelectrically focused using an Ettan IPGphor 3 (GE Healthcare, USA) system at 500 V for 1.5 hours, then ramped to 1000V for 1.0 hour, then ramped to 10,000 V for 3.5 hours and kept at 10,000 V for 11 hours.

Prior to the second dimension separation, disulfide bonds were reduced by incubating the immobilized DryStrips with 1% DTT in equilibration buffer for 15 minutes (7 M urea, 2 M thiourea, 2% SDS, 50 mM Tris-HCl, 30% glycerol, 0.002% bromophenol blue), after which free SH-groups were alkylated by treatment with 2.5% iodoacetamide in equilibration buffer. The strips were then transferred to 12.5% polyacrylamide gels (25×20 cm) and subjected to second dimensional electrophoresis (step 1: 10 mA/gel run for 1 hour, step 2: 40 mV/gel run for 5 hours). Gels were then fixed and stained by the nitrate silver staining method.

Quantification of Protein Expression: the stained gels were scanned on a Umax Powerlook 2100XL Scanner and analyzed using the Image Master 2-D Platinum Program. Protein spots on the gels were identified basic on pI and the molecular weight (MW) of the maker proteins. In electrophoresis, 2DE standard marker proteins were load together with samples. From MW & pI of marker proteins, we identified other protein's and determined proteins that were interested. Accuracy of proteins was ensured by the observation on 3D images of spots. The spot volume of interested proteins in the gel performed test samples was compared to spot volume of equivalent proteins in the gel performed control.

3. RESULTS AND DISCUSSION

MMP-1, which is known as collagenase-1, is one of the most important enzymes in collagen degradation in skin. Substrates of MMP-1 include type I, II, III, VII, VIII, X collagen and gelatin. MMP-1 preferentially cleaves type III collagen [10]. Active ingredients that inhibit MMP-1 can inhibit skin degradation and have good effects in skin anti-aging. In this experi-

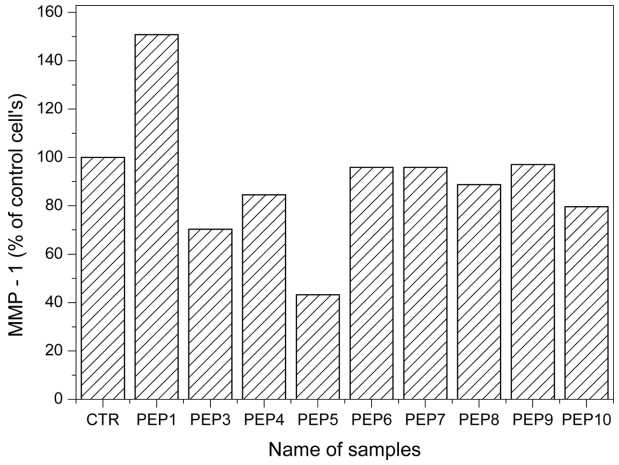

Fig. 1. The comparative expression of MMP-1 in human dermal fibroblasts treated with various peptide fractions of *A. membranaceus* separated by electrophoresis. CTR means the control without treatment. PEP1 to PEP10 are human dermal fibroblasts treated with peptides fractions 1 to 10 separated by electrophoresis with 40% polyacrylamide gel.

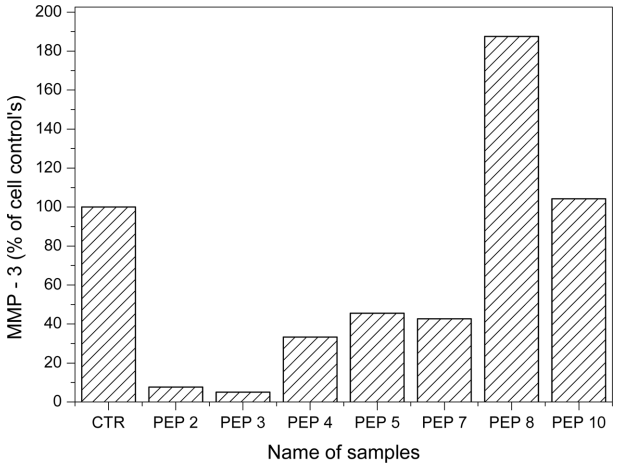

Fig. 2. The comparative expression of MMP-3 in human dermal fibroblasts treated with various peptide fractions of *A. membranaceus* separated by electrophoresis.

ment, MMP-1 in the test sample and control was identified (Fig. 1). Except for sample that treated with AMPs separated from portion 1 of the 40% acrylamide gel, MMP-1 reduced in all of the human dermal fibroblasts treated with AMPs relative to the control. Test samples treated with AMPs separated from portion 5 of the 40% acrylamide gel showed the strongest reduction. MMP-1 in this case was reduced to 43.2% relative to the control. These findings indicate that AMPs, especially, which separated from portion 5 of the 40% acrylamide gel, led to down-regulation of MMP-1.

Effects of AMPs on MMP-3 regulation are shown in Fig. 2. MMP-3 of human dermal fibroblasts treated with AMPs separated on parts 2-7 of the 40% polyacrylamide gel was present in lower levels relative to untreated control cells. The greatest reduction was observed in human dermal fibroblasts treated with AMPs separated from portion 2 (7.6%) and portion 3 (5%) of the 40% acrylamide gel. MMP-3, which is known as stromelysin-1, activates some pro-MMPs during extracellular matrix turnover. The substrates of MMP-3 include gelatin and collagens III, IV, V, IX, X, and XI. MMP-3 efficiently activates collagenases, matrilysin, and gelatinase B, and the action of MMP-3 on partially processed pro-MMP-1 is critical to the generation of fully active MMP-1. Moreover, MMP-3 activates TGF-a, which then stimulates MMP-9 expression by macrophages in a COX-2 dependent manner [10]. Then, the active ingredients inhibit the expression of MMP-3, are the potential to inhibit skin degradation. Consequently, AMPs inhibit the expression of MMP-3, indicating that its inhibit skin degradation and has good anti-aging effect.

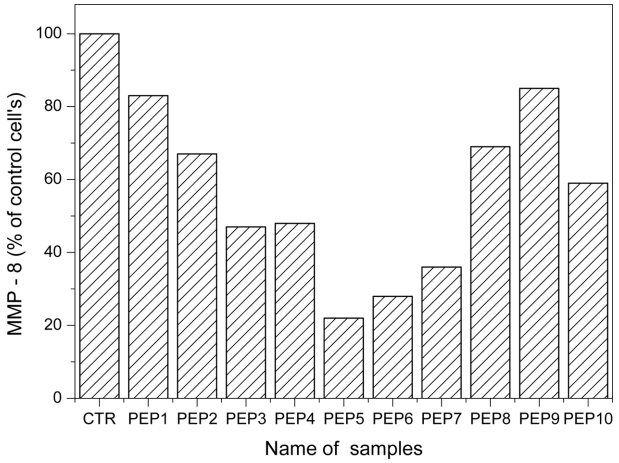

Fig. 3. The comparative expression of MMP-8 in human dermal fibroblasts treated with various peptide fractions of *A. membranaceus* separated by electrophoresis.

MMP-8, which is also known as collagenase-2 or neutrophil collagenase, is one of the major collagenases responsible for catalysis of the degradation of collagen in skin. MMP-8 has the greatest efficiency toward collagen I, which is a major component in skin [10]. In the present study, MMP-8 tended to decrease in human dermal fibroblasts treated with AMPs (Fig. 3). MMP-8 of human dermal fibroblasts treated with AMPs decreased relative to control cells, with the greatest reduction of 22% relative to the control being observed in human dermal fibroblasts treated with AMPs separated from portion 5 of the 40% polyacrylamide gel. These findings indicate that AMPs strongly inhibit the expression of MMP-8, which may lead to efficient inhibition of skin degradation and skin anti-aging.

Effect of AMPs on MMP-13 expression is shown in Fig. 4.

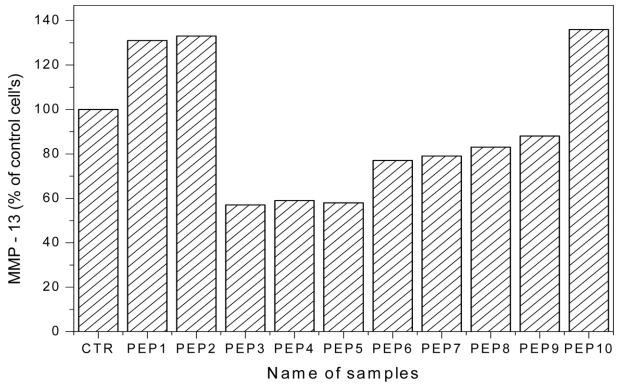

Fig. 4. The comparative expression of MMP-13 in human dermal fibroblasts treated with various peptide fractions of *A. membranaceus* separated by electrophoresis.

The results revealed that AMPs separated from portion 3 to portion 9 of the 40% polyacrylamide gel inhibited the expression of MMP-13. The best reductions of 57%, 58% and 59% were observed in human dermal fibroblasts treated with AMPs separated from portions 3, 4, and 5 of the gel, respectively. These findings indicated that AMPs inhibit the expression of MMP-13. MMP-13, which is also known as collagenase-3, preferentially hydrolyzes type II collagen, the major type of collagen found in cartilage. It should be noted that the gelatinolytic activity of MMP-13 is about 44 times greater than that of MMP-1, and that it plays an important role in extra-cellulose matrix degradation [10].

Techniques such as ultracentrifugation, molecular cut-off filtration, ultrafiltration and chromatography were needed to separate small peptides (ultra-peptides), especially those with molecular weights <1500 Da [11]. Additionally, these peptides could be separated by using urea-sodium dodecyl sulfate (SDS) - polyacrylamide gels [12] or tricine - SDS - polyacrylamide gels [13]. In our experiment, we only used 40% polyacrylamide gel without SDS to separate small peptides following the MW. This method is rapid, simple and it can apply in normal biotechnological laboratories.

Collagen is the major component of human skin, accounting for about 40% of the weight of dry skin. Collagen has important function in skin elasticity. In general, collagen degradation depends primarily on MMPs [14], but only a limited number of MMP family members (MMP-1 , MMP-8, MMP-13) can degrade interstitial collagen [15,16]. During aging of the skin, disequilibrium occurs in the balance between synthesis and degradation of collagen, leading to excessive collagen degradation [17]. Many reports indicated that MMPs accelerated skin aging, so active ingredients that inhibit the expression of MMPs have the potential to prevent skin aging [18,19]. In the present study indicated that AMPs inbibited the expression of MMP-1, MMP-3 and MMP-13. These were potential to apply in cosmetic formula of skin anti-aging.

Small peptides can easily penetrate cells and have good bioavailability [20]. The skin permeation of peptides increases as their molecular weight decreases [21]. In this experiment, we investigated the mass of AMPs separated on portion 5 of 40% polyacrylamide gel and found that it was < 1500 Da (Fig. 5). Subsequent evaluation of the effects of these peptides revealed that they had good effects leading to down regulation of MMPs

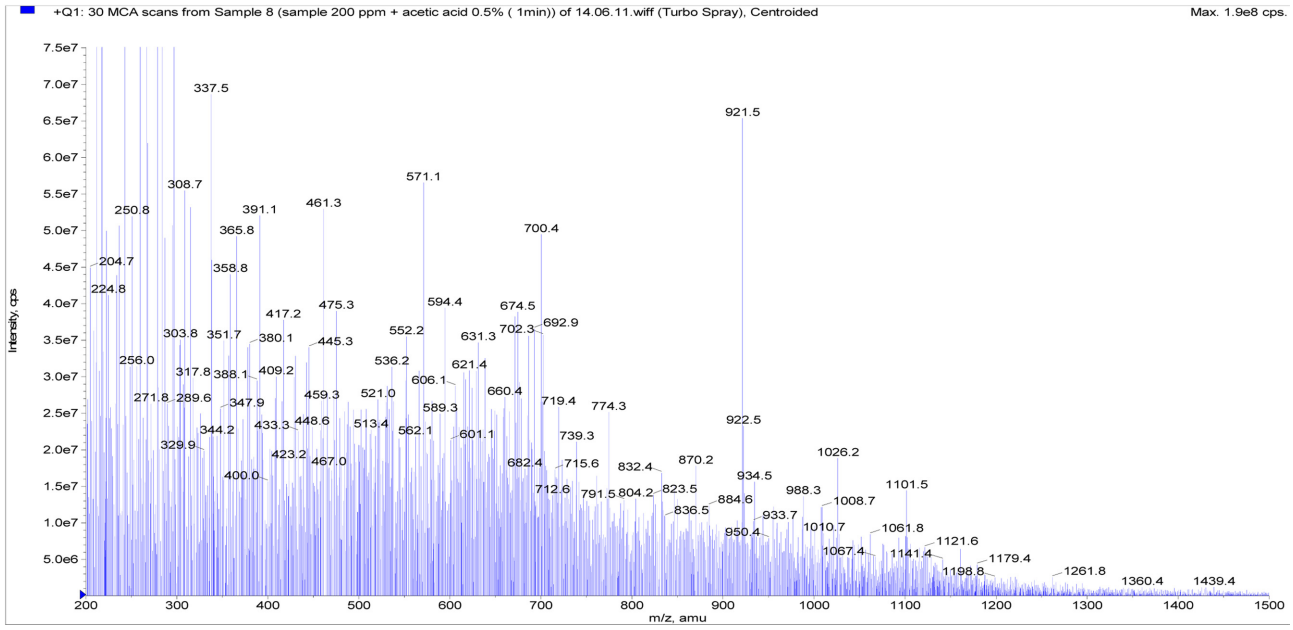

Fig. 5. The mass spectrometric analysis of AMPs from the fraction 5 separated by electrophoresis with 40% polyacrylamide gel.

and therefore have the potential for application to preparation of raw material for skin anti-aging cosmetics.

4. CONCLUSIONS

Peptide hydrolysates from *A. membranaceus* were found to inhibit expressions of MMPs in human dermal fibroblasts, which related with skin degradation. Inhibition effects were increased by peptides purified with electrophoresis. Expressions of MMP-1, MMP-3, MMP-8 and MMP-13 were reduced in 43%, 5%, 22% and 57% respectively compared to control cells without treatment of *AMP*. The mass spectrometric analysis of partially purified peptides from *A. membranaceus* in portion 5 of electrophoresis gels, indicated that almost peptide hydrolysates were composed of molecules less than 1500 Da.

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