

# ***Pyropia yezoensis* peptide promotes collagen synthesis by activating the TGF- $\beta$ /Smad signaling pathway in the human dermal fibroblast cell line Hs27**

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**Abstract.** *Pyropia yezoensis* (*P. yezoensis*) is a marine algae that exhibits antioxidant, anti-inflammatory, antitumor and anti-aging activities. In this study, we investigated the effects of the *P. yezoensis* peptide, PYP1-5, on collagen synthesis in the human dermal fibroblast cell line Hs27. Skin aging is related to reduced collagen production and the activities of multiple enzymes, including matrix metalloproteinases (MMPs), which degrade collagen structure in the dermis, and tissue inhibitor of metalloproteinases (TIMPs), which inhibit the action of MMPs. While collagen synthesis is associated with a number of signaling pathways, we examined the increased collagen synthesis via the upregulation of the transforming growth factor- $\beta$  (TGF- $\beta$ )/Smad signaling pathway. Using MTS assay, we found that PYP1-5 did not affect cell viability. Moreover, we confirmed that PYP1-5 increased type 1 collagen expression using enzyme-linked immunosorbent assay (ELISA), western blot analysis and quantitative PCR. In addition, we identified changes in various enzymes, as well as the mechanisms behind the PYP1-5-induced collagen synthesis. PYP1-5 decreased the MMP-1 protein and mRNA levels, and increased the TIMP-1 and TIMP-2 protein and mRNA levels. In addition, PYP1-5 activated the TGF- $\beta$ /Smad signaling pathway, which increased TGF- $\beta$ 1, p-Smad2 and p-Smad3 expression, while inhibiting Smad7, an inhibitor of the TGF- $\beta$ /Smad pathway. Furthermore, PYP1-5 upregulated transcription factor specificity protein 1 (Sp1) expression, which is reportedly involved in type 1 collagen expression. These findings indicate that PYP1-5 activates the TGF- $\beta$ /Smad signaling pathway, which subsequently induces collagen synthesis in Hs27 cells.

## **Introduction**

In recent years, the average human lifespan has increased due to economic growth and advancements in modern medicine. As a result, people have begun to pay more attention to skin health and beauty (1). Many research efforts have focused on the identification of strategies with which to inhibit and delay skin aging, and many cosmetic and food products related to skin anti-aging have been developed (2).

Skin aging is driven by intrinsic and extrinsic factors that cause structural degradation and alterations. Intrinsic aging occurs naturally with aging, while extrinsic aging is caused by external factors, such as ultraviolet (UV) radiation, gravity and air pollution (3). The characteristics of intrinsic aging include reduced levels of extracellular matrix components, such as collagen and elastin, which decrease skin elasticity and tension (4). Therefore, maintaining collagen levels in the dermis is important for maintaining healthy skin. Skin is composed of various layers, including the epidermis composed of epithelial tissue and the dermis composed of connective tissue and subcutaneous layer. The epidermis protects the skin from microbial pathogens, UV light and chemical compounds. The dermis is composed of fibrous proteins, including ground substance, collagen and elastin. It is an important layer that constitutes >90% of the skin (5). A variety of substrates, including collagen and elastin in the extracellular matrix (ECM), are made by dermal fibroblasts. There are several types of collagen, which accounts for 80-90% of the dermis. Type 1 collagen constitutes approximately 85% of total collagen and provides tension, elasticity and flexibility to the skin as it is entangled in elastic fibers (6). Its structure is maintained by several enzymes; matrix metalloproteinases (MMPs) secreted by fibroblasts degrade collagen, while MMP activity is inhibited by tissue inhibitor of metalloproteinases (TIMPs). During the aging process, MMP expression gradually increases and TIMP expression decreases, promoting collagen degradation and reducing skin elasticity (7,8).

Transforming growth factor- $\beta$  (TGF- $\beta$ ) is a multifunctional cytokine with three isoforms, TGF- $\beta$ 1, TGF- $\beta$ 2 and TGF- $\beta$ 3. TGF- $\beta$  helps regulate cellular processes, such as cell growth, differentiation, migration, apoptosis and the production of

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various ECM components, including collagen, elastin and fibronectin (9,10). TGF- $\beta$  interacts with two types of receptors containing type 1 and 2 receptors (serine/threonine kinase receptors). To activate the TGF- $\beta$ /Smad signaling pathway, the TGF- $\beta$ 1 ligand first binds to type 2 receptors (TGF- $\beta$ RII) at the cell surface, which allows for the phosphorylation of the GS domain of TGF- $\beta$ R1, activating downstream signaling. The complex then enters the nucleus from the cytoplasm, where it can regulate the expression of target genes by binding to promoters and co-factors to activate transcription (11,12).

Recent studies have found that marine algae, including red, brown and green algae, are rich in nutrients with a variety of bioactive functions. For example, *Pyropia yezoensis* (*P. yezoensis*), a red alga, is cultivated abundantly in East Asian countries, including China, Japan and Korea (13). *P. yezoensis* is composed of 25-40% carbohydrates and 25-50% proteins based on its dry weight, and is a good source of physiologically active substances (14). *P. yezoensis* has numerous biological functions, including antioxidant, antitumor, anti-fatigue and anti-inflammatory activities, and has been shown to reduce blood pressure and protect against UVA-induced photo-aging (15-18). Although a number of studies are in progress to examine the biological effects of *P. yezoensis*, no studies have yet examined its effects against skin aging using human dermal fibroblasts, at least to the best of our knowledge. In this study, we found that the *P. yezoensis* peptide, PYP1-5, affected collagen synthesis in Hs27 cells. Furthermore, we determined the intracellular mechanisms responsible for PYP1-5 induced-collagen synthesis, focusing on the TGF- $\beta$ /Smad signaling pathway and enzymes related to collagen expression.

## Materials and methods

**Preparation of *P. yezoensis* peptide PYP1-5.** PYP1-5 (D-P-K-G-K-Q-Q-A-I-H-V-A-P-S-F) was prepared as described previously (19). The 15 N-terminal residues of PYP1-5 were synthesized by Peptron (Daejeon, Korea). PYP1-5 was purified using a Shimadzu Prominence high-performance liquid chromatography (HPLC) apparatus and the software package Class-VP version 6.14 (Shimadzu, Kyoto, Japan), with a C18 column (Capcell Pak; Shiseido, Tokyo, Japan) in 0.1% trifluoroacetic acid (TFA)/water, a gradient of 10-70% acetonitrile (0-20% acetonitrile for 2 min, 20-50% acetonitrile for 10 min, and 50-80% acetonitrile for 2 min) in 0.1% TFA, a flow rate of 1.0 ml/min, and UV detection at 220 nm. The molecular mass of PYP1-5 was confirmed to be 1,622 kDa based on mass spectrometry (HP 110 Series LC/MSD).

**Cell culture.** The human skin fibroblast cell line Hs27, was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in complete Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS; HyClone, Logan, UT, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified 5% CO<sub>2</sub> incubator at 37°C. The Hs27 cells were cultured to 70-80% confluence in a 100-mm diameter plate and were used between passage numbers 5 and 15.

**MTS assay.** Hs27 cell viability was estimated using a CellTiter 96 Aqueous Nonradioactive Cell Proliferation

assay (Promega, Madison, WI, USA). The cells were plated in 48-well plates at a density of  $2 \times 10^4$  cells/well, and subsequently treated with PYP1-5 (250, 500 and 1,000 ng/ml) in serum-free medium (SFM) for 24 h. The cells were then incubated with 10  $\mu$ l of MTS solution for 30 min at 37°C, and the absorbance was quantified spectroscopically at 490 nm using a microplate reader (Benchmark microplate reader; Bio-Rad Laboratories, Hercules, CA, USA). Cell viability was calculated as the ratio of absorbance of treated cells to that of untreated cells.

**Procollagen type I C-peptide (PIP) EIA assay.** Procollagen was measured with a PIP EIA assay kit (Takara Bio Inc., Tokyo, Japan). The cells were inoculated in 6-well plates at a density of  $1 \times 10^5$  cells/well and incubated for 24 h in SFM containing PYP1-5 (250, 500 and 1,000 ng/ml). After 24 h, the supernatant was collected from each well and tested with the PIP EIA kit following the manufacturer's instructions.

**Treatment with TGF- $\beta$ R1 inhibitor (SB431542).** The cells were incubated for 2 h with 10  $\mu$ M SB431542 (Tocris, Bristol, UK) prior to treatment with PYP1-5.

**Whole-cell protein lysate extraction.** The Hs27 cells were seeded in 100-mm dishes and cultured to 80% confluence. The cells were treated for 24 h with PYP1-5 (250, 500 and 1,000 ng/ml), washed 2 times in phosphate-buffered saline, and scraped on ice in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 2 mM EDTA) containing protease inhibitor (1 mg/ml aprotinin, 1 mg/ml leupeptin, 1 mg/ml pepstatin A, 200 mM Na<sub>3</sub>VO<sub>4</sub>, 500 mM NaF and 100 mM phenylmethylsulfonyl fluoride). The cell extracts were centrifuged at 14,000 rpm for 10 min, and the supernatant was collected for use in western blot analysis.

**Western blot analysis.** Sample proteins (30  $\mu$ g) were separated with 5-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 1% bovine serum albumin in TBS-T (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween-20), probed with specific primary antibodies, and then probed with the secondary antibodies. Signals were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc., Rockford, IL, USA). The following primary and secondary antibodies were used: anti-collagen I (COL-1; sc-59772, anti-mouse, 1:1,000), anti-elastin (sc-166543, anti-mouse, 1:1,000), anti-MMP-1 (sc-21731, anti-mouse, 1:1,000), anti-TIMP-1 (sc-5538, anti-rabbit, 1:2,000), anti-TIMP-2 (sc-5539, anti-rabbit, 1:2,000), anti-TGF- $\beta$ 1 (sc-146, anti-rabbit, 1:1,000), anti-p-Smad2 (sc-135644, anti-rabbit, 1:500), anti-Smad2 (sc-6200, anti-goat, 1:1,000), anti-p-Smad3 (sc-130218, anti-rabbit, 1:500), anti-Smad7 (sc-11392, anti-rabbit, 1:2,000) and anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (sc-25778, anti-rabbit, 1:2,000) (all from Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), anti-Smad3 (#9513, anti-rabbit, 1:1,000; Cell Signaling Technology, Danvers, MA, USA), donkey anti-goat IgG (A50-101P, 1:10,000; Bethyl Laboratories, Inc., Montgomery, TX, USA),

Table I. The oligonucleotide primer sequences used in the real-time PCR.

| Gene                           | Accession no.  | Sequence (5'-3')  | Amplicon (bp) |
|--------------------------------|----------------|---|---------------|
| <i>COL1A1</i>                  | NM_000088.3    | F: AGGGCCAAGACGAAGACATC<br>R: AGATCACGTCATCGCACAACA       | 138           |
| <i>COL1A2</i>                  | NM_000089.3    | F: TCTGGATGGATTGAAGGGACA<br>R: CCAACACGTCTCTCTCACC        | 126           |
| <i>Elastin</i>                 | XM_011515875.1 | F: ATGCACACTGGTGCAGAGAG<br>R: TGTAAGCACACAGGCAGGTC        | 98            |
| <i>TGF-<math>\beta</math>1</i> | NM_000660.5    | F: AGCGACTCGCCAGAGTGGTTA<br>R: GCAGTGTGTTATCCCTGCTGTCA    | 130           |
| <i>MMP-1</i>                   | NM_002421.3    | F: CCCAAAAGCGTGTGACAGTAAG<br>R: CTTCCGGGTAGAAAGGGATTG     | 113           |
| <i>TIMP-1</i>                  | NM_003254.2    | F: TGACATCCGGTTCGTCTACA<br>R: TGCAGTTTTCCAGCAATGAG        | 102           |
| <i>TIMP-2</i>                  | NM_003255.4    | F: GCGGTCAGTGAGAAGGAAGTGGA<br>R: GAGGAGGGGGCCGTGTAGATAAAC | 140           |
| <i>GAPDH</i>                   | NM_001256799.2 | F: ACCCACTCCTCCACCTTTGA<br>R: TGGTGGTCCAGGGGTCTTAC        | 157           |

COL1A1, collagen type I  $\alpha$ 1; TGF- $\beta$ 1, transforming growth factor- $\beta$ 1; MMP-1, matrix metalloproteinase-1; TIMP-1, tissue inhibitor of metalloproteinase-1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

goat anti-mouse IgG-HRP (sc-2031, 1:10,000; Santa Cruz Biotechnology, Inc) and goat anti-rabbit IgG (#7074, 1:10,000; Cell Signaling Technology).

**Quantitative PCR (qPCR).** The Hs27 cells seeded in 6-well plates were cultured to 80% confluence and incubated in SFM containing PYP1-5 (250, 500 and 1,000 ng/ml) for 24 h. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and the extracted RNA was used as a template for cDNA synthesis using oligo(dT) (Intron Biotechnology Inc., Seongnam, Korea). The PCR amplification mixtures (total volume, 20  $\mu$ l) contained 10  $\mu$ l of TOPreal qPCR 2X PreMIX SYBR-Green (Enzynomics Inc., Daejeon, Korea), 1  $\mu$ l of sense primer, 1  $\mu$ l of anti-sense primer, 2  $\mu$ l of cDNA template, and 6  $\mu$ l of RNase-free water. qPCR was performed using the Eco Real-Time PCR system (Illumina Inc., San Diego, CA, USA) using the following amplification conditions: pre-incubation at 95°C for 10 min, 40 cycles of 95°C for 10 sec, annealing at 60°C for 15 sec, and elongation at 72°C for 15 sec. Gene expression levels were normalized to those of GAPDH and calculated using the comparative  $\Delta\Delta C_T$  method, as previously described (20). The oligonucleotide primers used for PCR are listed in Table I.

**Statistical analysis.** All samples were analyzed in triplicate. The results are expressed as the means  $\pm$  standard deviation (SD). To determine statistical significance, analysis of variance (ANOVA) was conducted using SPSS software (SPSS, Inc., Chicago, IL, USA). A value of  $p < 0.05$  was considered to indicate a statistically significant difference.

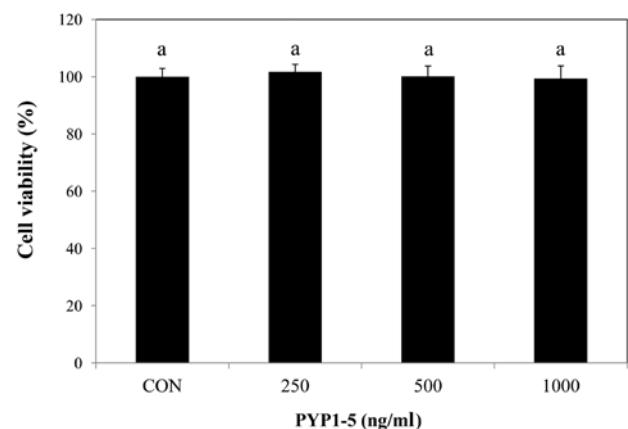


Figure 1. Cytotoxicity of PYP1-5 to Hs27 cells. The cells were seeded in 48-well plates at a density of  $2 \times 10^4$  cells/well with medium supplemented with 10% FBS. Following incubation, the cells were treated with PYP1-5 (250, 500 and 1,000 ng/ml) for 24 h. Cell viability was determined by MTS assay. The results are presented as the means  $\pm$  SD of 3 independent experiments. Bars labeled with the same letters indicate no statistical significance.

## Results

**Effects of PYP1-5 on Hs27 cell viability.** We used MTS assay to determine PYP1-5 cytotoxicity to Hs27 cells. Fig. 1 shows the effects of various concentrations (250, 500 and 1,000 ng/ml) of PYP1-5 on Hs27 cell viability. The PYP1-5-treated cells exhibited similar effects on viability at concentrations of 250, 500 and 1,000 ng/ml as the untreated cells and had no significant cytotoxicity.

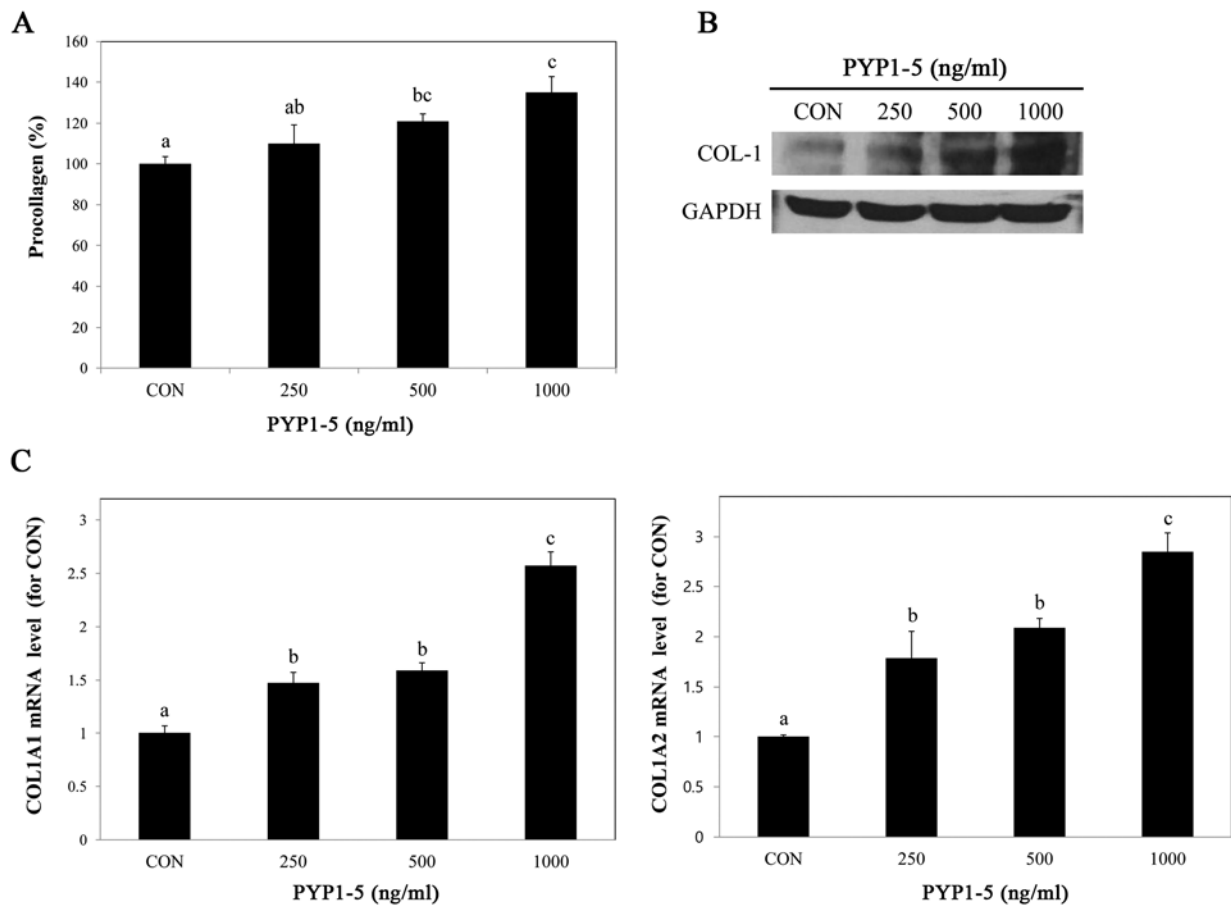


Figure 2. Effect of PYP1-5 on the expression of type I collagen in Hs27 cells. (A) Procollagen expression was investigated using the PIP EIA kit assay. (B) Type I collagen protein expression was examined with a western blot analysis. (C) Collagen type I  $\alpha 1$  (*COL1A1*) and *COL1A2* mRNA levels were examined by qPCR. The results are presented as the means  $\pm$  SD of 3 independent experiments. Bars labeled with different letters indicate statistically significant differences ( $p < 0.05$ ).

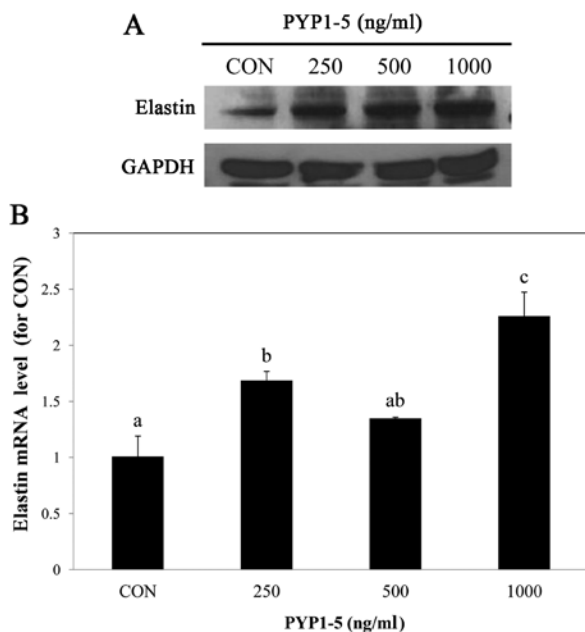


Figure 3. Effect of PYP1-5 on elastin expression in Hs27 cells. The cells were cultured in 100-mm dishes with PYP1-5 (250, 500 and 1,000 ng/ml) for 24 h, after which cellular protein and total RNA were isolated. (A) Protein expression levels were assessed by western blot analysis. (B) mRNA expression levels were quantified by qPCR. The results are presented as the means  $\pm$  SD of 3 independent experiments. Bars labeled with different letters indicate statistically significant differences ( $p < 0.05$ ).

*PYP1-5 promotes type I collagen synthesis.* To determine whether PYP1-5 affects collagen synthesis, we performed a PIP EIA assay. After treating the Hs27 cells with various concentrations of PYP1-5, we measured procollagen products in the cells and found that procollagen product levels increased in a dose-dependent manner (Fig. 2A). Using western blot analysis, we confirmed that the type 1 collagen protein expression levels were increased (Fig. 2B). Type 1 collagen is composed of two  $\alpha 1(I)$  chains and one  $\alpha 2(I)$  chain, which are encoded by two genes, *COL1A1* and *COL1A2*, respectively (21). We further investigated the above-mentioned by qPCR and found that both the *COL1A1* and *COL1A2* mRNA expression levels increased in a dose-dependent manner (Fig. 2C).

*PYP1-5 increases elastin expression.* Elastin is an important component of connective tissue, such as collagen and is located between collagen in the dermis, providing elasticity and flexibility to the skin. With age, elastin decomposes, resulting in reduced skin elasticity and increased skin aging (22,23). In this study, to examine the effect of PYP1-5 on elastin in Hs27 cells, we performed western blot analysis and qPCR and found that the elastin protein and mRNA expression levels increased following treatment with PYP1-5 (Fig. 3).

*PYP1-5 decreases MMP-1 expression and increases TIMP-1 and -2 expression.* To confirm the regulatory effects of PYP1-5

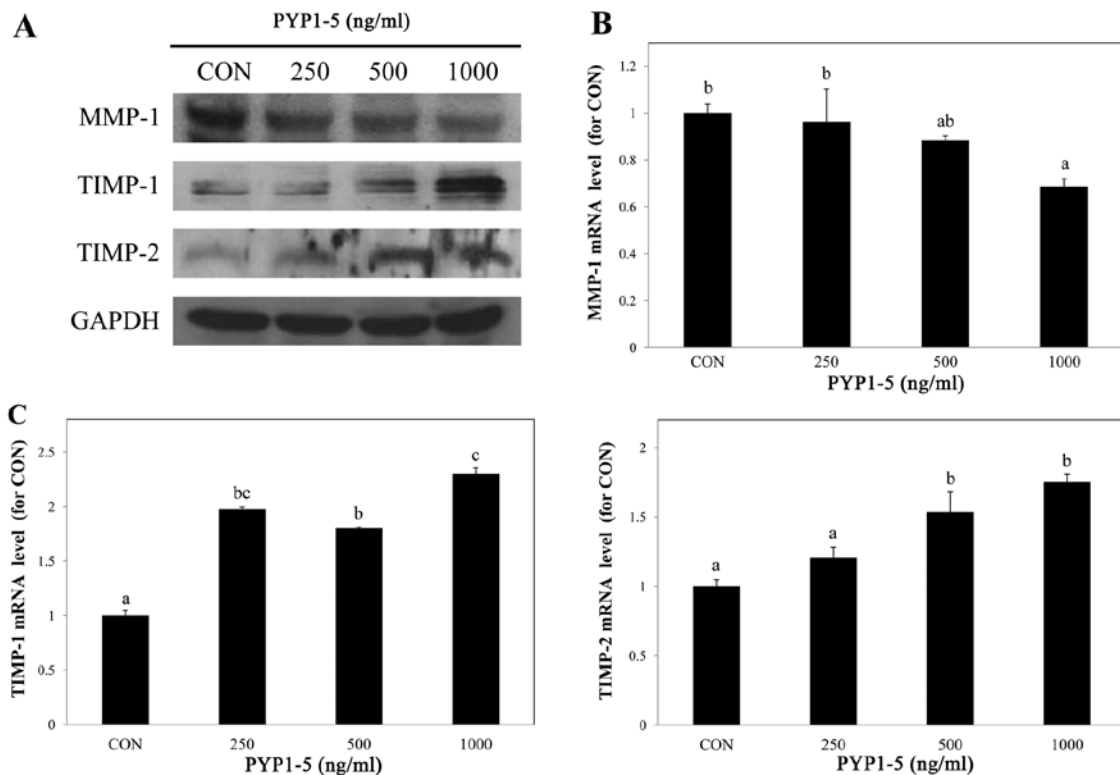


Figure 4. Effect of PYP1-5 on matrix metalloproteinase-1 (MMP-1), tissue inhibitor of metalloproteinase-1 (TIMP-1) and TIMP-2 protein and mRNA expression levels in Hs27 cells. (A) Protein expression levels were assessed by western blot analysis. (B and C) mRNA expression levels were quantified by qPCR. The results are presented as the means  $\pm$  SD of 3 independent experiments. Bars labeled with different letters indicate statistically significant differences ( $p < 0.05$ ).

on ECM synthesis enzymes, we examined MMP-1 and TIMP-1 and -2 protein and mRNA expression by western blot analysis and qPCR, respectively. Following treatment of the cells with PYP1-5 for 24 h, the MMP-1 protein and mRNA expression levels decreased in a dose-dependent manner (Fig. 4A and B), while the TIMP-1 and -2 protein and mRNA expression levels increased (Fig. 4A and C). These results indicate that PYP1-5 regulates collagen synthesis by reducing MMP-1 expression and inducing TIMP-1 and -2 expression.

*PYP1-5 activates the TGF- $\beta$ /Smad signaling pathway.* We then investigated the mechanisms through which PYP1-5 promotes type 1 collagen expression in Hs27 cells. Since the TGF- $\beta$ /Smad pathway is the major signaling pathway of collagen synthesis in the dermis (24), we assessed whether it is involved in PYP1-5-induced type 1 collagen expression.

To investigate the regulatory effects of PYP1-5 on the TGF- $\beta$ /Smad signaling pathway, we measured downstream protein and mRNA expression levels in Hs27 cells using by western blot analysis and qPCR, respectively. We confirmed that PYP1-5 increased TGF- $\beta$ 1 ligand protein and mRNA expression levels in a dose-dependent manner (Fig. 5A and B). In addition, treatment with PYP1-5 increased the TGF- $\beta$ RII and p-Smad2/3 protein levels. Treatment of the Hs27 cells with PYP1-5 also decreased the Smad7 protein levels, an inhibitor of the TGF- $\beta$ /Smad signaling pathway (Fig. 5C). These results indicate that treatment of the Hs27 cells with PYP1-5 activates the TGF- $\beta$ /Smad signaling pathway. To further investigate the involvement of the TGF- $\beta$ /Smad pathway in PYP1-5-induced collagen synthesis, we used a specific inhibitor of TGF- $\beta$ RI,

SB431542 (10  $\mu$ M). Treatment with SB431542 downregulated the PYP1-5-induced upregulation of p-Smad2/3, a downstream target of TGF- $\beta$ /Smad signaling (Fig. 6A) and attenuated the effects of PYP1-5 on the MMP-1 protein, and *TIMP-1* and *TIMP-2* mRNA levels (Fig. 6A and B). In addition, treatment with SB431542 reduced the PYP1-5-induced *COL1A1* and *COL1A2* mRNA levels (Fig. 6C). These data suggest that PYP1-5 regulates collagen synthesis via the TGF- $\beta$ /Smad signaling pathway.

*PYP1-5 increases the expression of specificity protein 1 (Sp1) transcription factor via the TGF- $\beta$ /Smad signaling pathway.* Sp1 is an essential zinc finger transcription factor of Smad-dependent positive regulation of collagen synthesis that is induced by TGF- $\beta$ . The Sp1 transcription factor regulates multiple biological processes, such as tumorigenesis, apoptosis, cell cycle and angiogenesis, and induces type I procollagen synthesis in fibroblasts (25). The Smad3/4 complex induces the trans-activation of the human *COL1A1* and *COL1A2* promoter in normal skin fibroblasts (26,27). In this study, we confirmed the effect of PYP1-5 on Sp1 protein levels induced by the TGF- $\beta$ /Smad signaling pathway and found that treatment with PYP1-5 increased Sp1 protein expression (Fig. 7).

## Discussion

Interest in anti-aging has grown with the increase in life expectancy. In particular, many efforts have been made to prevent skin aging (28) and much research has been dedicated to skin health and beauty. Skin aging is classified as either intrinsic or extrinsic. Intrinsic aging occurs due to a reduction in the

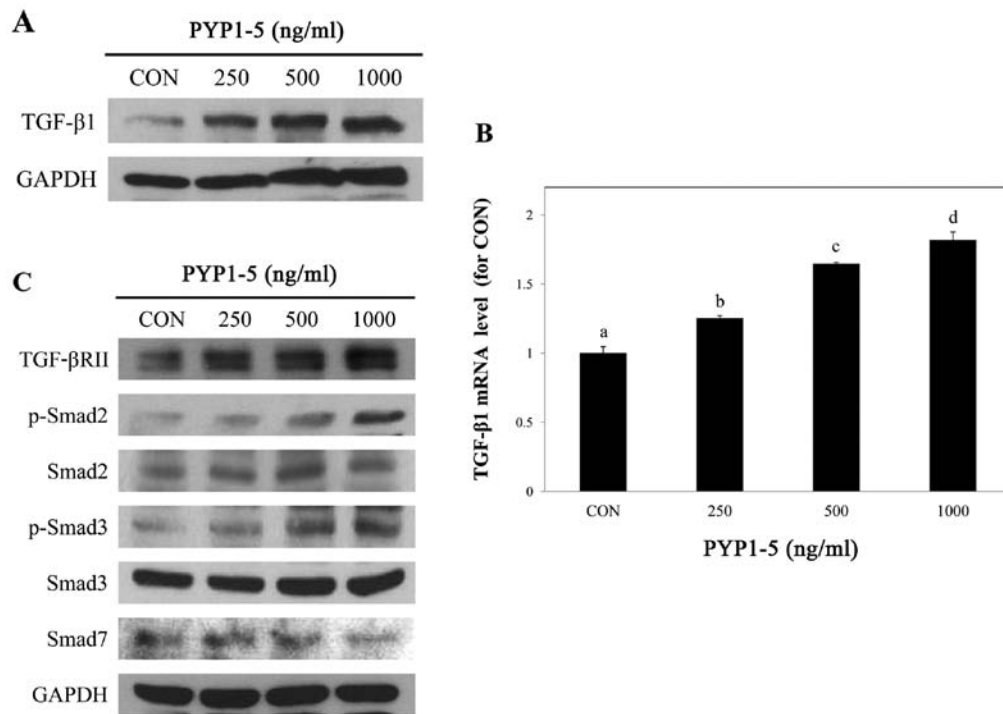


Figure 5. PYP1-5 activation of the transforming growth factor- $\beta$  (TGF- $\beta$ )/Smad signaling pathway in Hs27 cells. (A-C) Protein expression levels were assessed by western blot analysis and the mRNA expression levels were quantified by qPCR. The results are presented as the means  $\pm$  SD of 3 independent experiments. Bars labeled with different letters indicate statistically significant differences ( $p < 0.05$ ).

levels of ECM components, such as collagen and elastin, with age (29). Since collagen, the predominant component of the dermis, must be maintained to prevent the skin aging process, it is important to prevent its decomposition.

Various bioactive ingredients have been identified in many marine algae species, several of which have been studied for their anti-aging effects on skin, including anti-photoaging, anti-free radical activity, moisturization, and collagen biosynthesis (30). It has been demonstrated that *P. yezoensis* extract exerts a protective effect against UVA radiation on skin fibroblasts (18). However, the effects of *P. yezoensis* on collagen synthesis in human dermal fibroblasts remain unclear. In this study, we examined the *P. yezoensis* peptide, PYP1-5, for its anti-aging function by promoting collagen synthesis in human dermal fibroblasts. First, we investigated whether PYP1-5 induced cytotoxicity in Hs27 cells. We found that the PYP1-5-treated cells had a similar viability as the untreated cells (Fig. 1), suggesting that PYP1-5 was non-toxic to Hs27 cells. In the dermis, collagen gradually decomposes due to extrinsic and intrinsic factors, which promotes wrinkle formation and skin aging (31). PYP1-5 increased procollagen synthesis in a dose-dependent manner, with a maximum increase of 35% more than the untreated cells (Fig. 2A). In addition, PYP1-5 upregulated type 1 collagen protein levels and *COL1A1* and *COL1A2* mRNA expression (Fig. 2B and C). These results indicate that PYP1-5 promotes collagen synthesis.

Elastin is another vital component of the skin, as it is a protein that is located between collagen in the dermis that provides elasticity and flexibility to the skin (23). Following the treatment of the Hs27 cells with PYP1-5, the elastin protein and mRNA expression levels increased (Fig. 3), suggesting that PYP1-5 promotes the synthesis of other ECM products.

Further research is required to elucidate its effects on other ECM components, such as hyaluronan and fibronectin. In the dermis, various MMP enzymes degrade collagen, while TIMP enzymes inhibit MMP activity (8). We confirmed that PYP1-5 suppressed the MMP-1 protein and mRNA expression levels (Fig. 4A and B), and enhanced the TIMP-1 and -2 protein and mRNA expression levels (Fig. 4A and C).

TGF- $\beta$  is the major activator of the collagen synthesis process in skin fibroblasts. The process is also related to the TGF- $\beta$ /Smad signaling pathway (32). PYP1-5 upregulated the TGF- $\beta$ 1 protein and mRNA levels in a dose-dependent manner (Fig. 5A and B). Moreover, we confirmed that PYP1-5 increased TGF- $\beta$ RII, which resulted in Smad2 and Smad3 phosphorylation (Fig. 5C). This forms a complex with Smad4, which can translocate to the nucleus. To confirm that PYP1-5-induced collagen synthesis was caused by TGF- $\beta$ /Smad signaling pathway activation, we treated the Hs27 cells with SB431542, a specific inhibitor of TGF- $\beta$ RI. Treatment with SB431542 decreased TGF- $\beta$ /Smad signaling pathway activation by PYP1-5 (Fig. 6A). In addition, PYP1-5-induced Smad2/3 activation was inhibited by SB431542, suggesting that the phosphorylation of Smad2/3 is mediated by PYP1-5 treatment. The effects of PYP1-5 on MMP-1 and TIMP-1 and -2 regulation were attenuated by treatment with SB431542 (Fig. 6A and B). Moreover, the PYP1-5-induced increase in *COL1A1* and *COL1A2* mRNA expression was decreased by treatment with SB431542 (Fig. 6C), indicating that PYP1-5-induced collagen synthesis is regulated by TGF- $\beta$ /Smad signaling pathway activation. The Sp1 transcription factor, which is induced by TGF- $\beta$ /Smad signaling pathway activation, regulates type I procollagen expression (26). In this study, following treatment with PYP1-5, Sp1 protein expression increased (Fig. 7). This

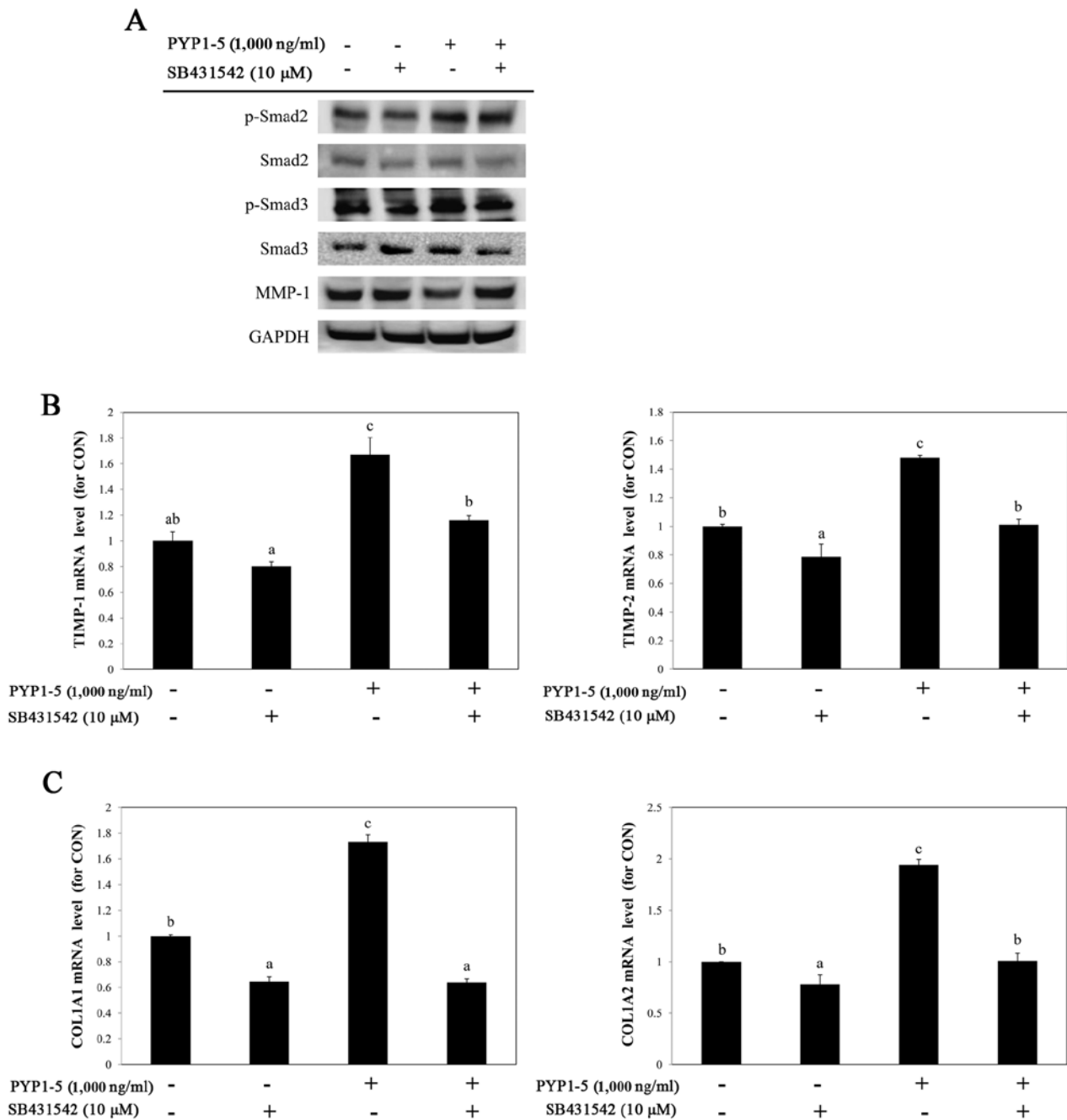


Figure 6. Effect of 10  $\mu$ M SB431542 on PYP1-5-induced collagen synthesis in Hs27 cells. (A) Protein expression was assessed by western blot analysis. (B and C) mRNA expression levels were quantified by qPCR. The results are presented as the means  $\pm$  SD of 3 independent experiments. Bars labeled with different letters indicate statistically significant differences ( $p < 0.05$ ).

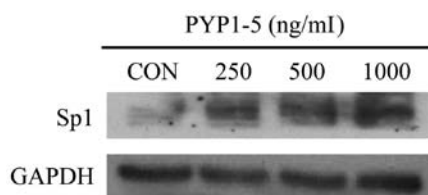


Figure 7. Effect of PYP1-5 on specificity protein 1 (Sp1) protein expression in Hs27 cells. Cells were treated with PYP1-5 for 24 h. Protein extracts were prepared and examined by western blot analysis.

In conclusion, the findings of this study demonstrate that PYP1-5 promotes type 1 collagen synthesis in Hs27 cells. Moreover, the TGF- $\beta$ /Smad signaling pathway may be important in mediating the effects of PYP1-5 on collagen synthesis. Our results suggest that PYP1-5 may have beneficial effects on intrinsic skin aging.

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demonstrates that the treatment of Hs27 cells with PYP1-5 affects transcription.



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