

Enhancement effects of sea mustard and starfish mixtures on skin activity

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미역 및 불가사리 추출 혼합물의 피부활성 증진 효능

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Abstract The enhancement effects of sea mustard extract (SME), starfish collagen peptide (SCP) and a mixture of the two (MIX) on skin activity were evaluated to investigate the possibility of using marine-derived extracts as cosmetic additives. The anti-inflammatory activity, whitening activity and skin elasticity activity of the extracts were analyzed to evaluate their skin-activating effects. Inhibiting the generation of nitric oxide (NO) and the tyrosinase and elastase inhibitory activities were assessed as the bio-markers for evaluating skin activity. SME, SCP and MIX did not show cytotoxicity within the concentration range of 1.0-50 µg/mL. In addition, SME, SCP and MIX all increased NO production and the tyrosinase and elastase inhibitory activities in a concentration-dependent manner. The activity of MIX was significantly increased compared to that with using SME or SCP alone. Taken together, when natural extracts are applied as cosmetic additives, the results demonstrate that using a mixture of SME and SCP may have a greater synergistic effect than that when using only a single extract. Therefore, this study contributes to the knowledge about the kinds and composition of several natural extracts when they are used as cosmetic additives.

요약 해양생물 추출물의 화장품 첨가제로서의 활용 가능성을 파악하고자, 미역 추출물, 불가사리 콜라겐 펩티드 및 혼합물의 피부 활성증진 효과를 평가하였다. 추출물의 피부 활성 증진 평가를 위하여 항염 활성, 미백효과 및 피부탄성 증진 활성을 분석하였다. 피부활성증진 평가를 위한 바이오마커로는 NO 생성 저해, 티로시나제 및 엘라스타제 저해 활성법을 적용하였다. 미역 추출물, 불가사리 콜라겐 펩티드 및 혼합물은 실험구간인 1.0-50 µg/mL까지 세포독성을 나타내지 않았다. 또한 미역 추출물, 불가사리 콜라겐 펩티드 및 혼합물은 농도의존적으로 NO 생성 저해, 티로시나제 및 엘라스타제 저해 활성이 증가하였다. 특히 혼합물을 사용하는 경우 미역 추출물 및 불가사리 콜라겐 펩티드를 단독으로 사용하는 것에 비하여 크게 활성이 증가하는 경향을 보였다. 종합해 보았을 때, 생물유래 천연추출물이 화장품 첨가제로 적용할 경우, 혼합물을 첨가하는 것이 단일 추출물로부터 얻을 수 있는 효과보다 더 좋은 시너지 효능이 있을 수 있다는 것을 알게 해준다. 따라서 본 연구의 결과는 천연추출물을 화장품 첨가제로서 활용하는 경우 추출물의 종류 및 배합 연구에 공헌할 수 있을 것이다.

Keywords : *Undaria Pinnatifida*, *Asterias Amurensis*, Nitric Oxide, Tyrosinase, Elastase

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1. Introduction

Seaweeds are widely distributed throughout the world, and they are used as food in East Asia, including Korea, China and Japan [1]. In Korea, about 800 species of algae are known to exist, of which about 50 species are used for food. Seaweeds have a low content of protein and fat, high carbohydrate content, and high content of various minerals and vitamin-A (β -carotene), vitamin-C and niacin. Therefore, seaweeds are widely used in various functional foods and biomaterials [2]. Functional materials derived from seaweeds have physiological properties such as anti-tumour, antiviral, anticoagulant and immunity-enhancing functions [3,4]. Among seaweed, brown algae contain a large number of water-soluble polysaccharides such as alginic acid, fucoidan and laminarin. Fucoidan contained in the sea mustard *Undaria pinnatifida* is a polysaccharide of about 200,000 Da. It contains a sulfate group and has been reported to have anti-tumour activity, immunity enhancement and skin moisturising activity [5-7].

The starfish *Asterias amurensis* is about 10-20 cm in diameter and lives in the sea-bottom sandy mud, which is about 100 m deep off the coast. It is widely distributed in the Northwest Pacific and is distributed in most coastal areas of Korea [8]. The collagen peptide derived from starfish is called hydrolysis gelatin, and its molecular weight is reduced to facilitate its absorption into the human body [9]. In previous studies, starfish peptides have been shown to enhance skin activity [10], to have anti-wrinkle activity [11], to protect skin against photodamage (anti-aging) [12], to enhance immune function [13] and to serve as an epidermal skin barrier [14].

In the last decades, studies on various physiological activities using seaweed extract and starfish collagen extract have been carried out, but research on the activity of mixtures has hardly been conducted. In this study, we

compared the physiological activities such as anti-inflammatory, whitening and wrinkle-reducing effects of seaweed extract, starfish collagen peptide and a mixture of the two. The results of this study show that a mixture of various biologically derived extracts can contribute to the knowledge of utilising functional food materials in cosmetics.

2. Materials and Methods

2.1 Preparation of hot water extract from sea mustard

Undaria pinnatifida used in this study was collected from Uljin, Gyeongbuk, Korea and transferred to the laboratory while keeping it at 4°C. The collected seaweeds were washed several times and then lyophilised. Ten times distilled water (DW) was added to the lyophilised seaweed, and the first extraction was performed at 60-65°C for 4 hr. Three times as much water was added to the seaweed residue, the supernatant was removed, and then the second extraction was performed at 60-65°C for 4 hr. The first and second extracts were mixed to prepare the final seaweed extract. The seaweed extract was frozen at -20°C, lyophilised at 1 mmHg high vacuum, and then powdered.

2.2 Preparation of collagen peptide from starfish

The starfish *Asterias amurensis* was collected from the coast of Pohang, Gyeongbuk, Korea and stored at -20°C until pretreatment. The intestines were removed from the starfish and washed several times in running water. The starfish was cut into 10 cm³-sized portions, and the saline was removed by soaking in distilled water for 12 hr. The starfish dried for 2 days, was pulverised, and 30 g of the pulverised material was immersed in 270 mL of a 2.5% NaOH aqueous

solution. After 12 hr of treatment, the supernatant was removed. The pulverised starfish material and 500 mL of DW were mixed in a reactor, and citric acid was added so that the final pH of the mixture was 6.0. 1.7 g of amino-peptidase (Peprotech, Korea) and 5 g of Triton x-100 (Sigma-Aldrich, Korea) were added and the mixture was hydrolysed at 47°C for 4 hr while stirring. The hydrolysis reaction was terminated after reacting for 20 min at 80°C. 1 L of DW was added to the hydrolysed pulverised product, and the hot water extraction was performed at 70°C for 7 hr. The hot water extraction was filtered (6 μ m) to obtain a starfish collagen peptide. The final extracted starfish collagen peptide had a gelling temperature of 30–40°C and an average molecular weight of 200–2,500 daltons. The seaweed extract and starfish collagen peptide extracts were mixed 1:1, and the mixture was used for cytotoxicity and activity experiments.

2.3 Cell cultures

The B16F10 murine melanoma cell line (ATCC LOT.60508145) and RAW 264.7 cell lines (ATCC1TIB-71™) were obtained from American Type Culture Collection (Mannasa, VA, USA) and cultured according to widely used methods [15,16]. The cells were maintained in 25–75 mL flasks containing Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated bovine serum (FBS, GIBCO BRL, MD, USA), 2 mM L-glutamine, 1% non-essential amino acids, 100 U/mL penicillin, and 100 mg/mL streptomycin, in monolayers at 37°C in a humidified atmosphere of 5% CO₂. The media was replenished every third day.

2.4 Cytotoxicity test

Cell viability in sea mustard extract (SME), starfish collagen peptide (SCP) and the mixture (MIX) was evaluated by following a conventional

MTT assay [17,18]. 100 μ L of a suspension of 5000 cells/well of B16-F10 melanocytes (ATCC LOT.60508145) was dispensed into a 96-well plate. The cells were pre-incubated for 24 hr at 37°C and 5% CO₂ to attach the cells to the well plates. In the blank wells, 100 μ L of DMEM medium containing no B16-F10 melanocytes was added. SME, SCP and MIX were diluted in DW to prepare samples with concentrations of 0.1 mg/mL, 1.0 mg/mL, and 5 mg/mL, and then treated with 10 μ L of each concentration. Cell-free medium and EtOH were used as a negative and positive control, respectively. 10 μ L of CCK-8 solution was added to each well, incubated for 1 hr, and then absorbance was measured at 450 nm. Cell viability was calculated as: survival rate (%) = $(A_{\text{sample}} - A_{\text{b}}) / (A_{\text{c}} - A_{\text{b}}) \times 100$, where A_{sample} is the absorbance of the sample, A_{b} is the absorbance of the blank and A_{c} is the absorbance of the negative control.

2.5 Determination of nitric oxide

The nitric oxide (NO) assay was used to analyse the anti-inflammatory activity of the extract [19,20]. RAW 264.7 cells were seeded in a 24-well plate containing DMEM medium and cultured at 37°C for 24 hr. Cultures were exchanged with fresh DMEM medium, and SME, SCP and MIX were diluted by concentration and 10 μ L of each concentration was used. After 1 hr, lipopolysaccharide (LPS) was treated at 0.2 μ g/mL and cultured at 37°C for 24 hr. 100 μ L of the culture supernatant and 100 μ L of Griess reagent were mixed and dispensed into a 96-well plate. After reacting at room temperature for about 10 min, the absorbance was measured at 540 nm. NO levels were determined by standard curve of nitrite (0.1–10 μ M).

2.6 Tyrosinase inhibitory activity

In order to evaluate the skin-whitening efficacy of SME, SCP and MIX, B16-F10

melanocytes were treated with various concentrations of the samples, then their tyrosinase inhibitory activity was measured [21,22]. 10 μ L of extracts were added to each well and incubated at 37°C and 5% CO₂ for 24 hr. Subsequently, 10 μ L of 1 mg/mL trypsin was added and allowed to react for 10 min, followed by centrifugation at 1000 rpm for about 2 min to obtain pellets. The resulting pellet was dissolved in 0.5 mL of 1% Triton X-100 PBS, and 0.5 mL of a 0.2% L-DOPA in 0.1 M sodium phosphate buffer was added. The cells were incubated at 37°C for 2 hr and absorbance was measured at 490 nm. Inhibition (%) = $\{(A_0 - A_1)/A_0\} \times 100$ where A_0 is the control absorbance and A_1 is the absorbance of the test group.

2.7 Elastase inhibitory activity

In order to examine the effect of SME, SCP and MIX on skin wrinkles, elastase inhibitory activity was measured using B16-F10 melanocytes [23]. 10 μ L of each sample was dispensed into each well. 50 mL of porcine pancreas elastase (10 μ g/mL) dissolved in 50 mM tris-HCl buffer (pH 8.6) was added to each well, then N-succinyl-(L-Ala)-3-p-nitroanilide (0.5 mg/mL) was added in an amount of 100 μ L each. After 20 min of reaction, the inhibition rate of elastase activity of B16-F10 melanocytes was measured. Elastase inhibitory activity was measured based on the absorbance reduction rate of the added sample and the non-added sample. Elastase inhibitory activity was calculated using the following equation: Inhibition (%) = $(1 - A_1/A_0) \times 100$, where A_0 is the control absorbance and A_1 is the absorbance of the test group.

2.8 Data analysis

All experiments were performed in triplicate, and the results are presented as means \pm SDs. Statistical analysis was performed using SPSS software, and the normality and homogeneity of

the data were confirmed using analysis of variance (ANOVA). The differences between the control and experimental groups were assessed using a one-way ANOVA (Tukey's multiple comparison test). Statistical significance was set as $p < 0.05$.

3. Results

3.1 Evaluation of cell viability

The cytotoxicity of sea mustard extract (SME), starfish collagen peptide (SCP) and mixture (MIX) was evaluated using the B16-F10 melanocyte cell line (Fig. 1). Cell viability was below 10% when the negative control was treated with ethanol. However, treatment with SME, SCP and MIX up to 50 μ g/mL showed a high cell survival rate of over 90%. These results indicate that SME, SCP and MIX are stable and do not affect cytotoxicity.

3.2 Inhibition of NO production

The effects of the extracts on nitric oxide production were analysed using the LPS-treated RAW 264.7 cell line (Fig. 2). When SME, SCP and MIX were treated with 0.2 μ g/mL of LPS for 24 hr, concentration- dependent inhibition of NO production was observed ($p < 0.05$). When 1.0 mg/mL of SME, SCP and MIX were treated, NO production inhibitory activity was hardly observed. When 10 μ g/mL of extract was treated, NO production inhibition activity was observed, and when 50 μ g/mL of MIX was treated, it was about 2.7 times more effective. In particular, the inhibitory effect of MIX at 50 μ g/mL was about 44% higher than with SME treatment and 26% higher than with SCP treatment alone.

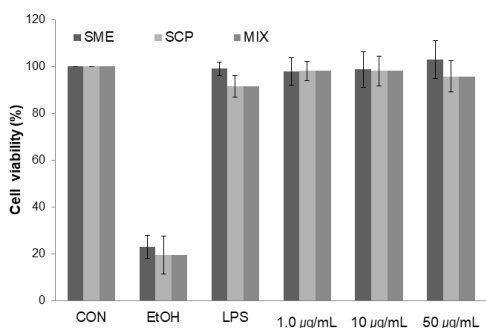


Fig. 1. Cytotoxicity of sea mustard extract (SME), starfish collagen peptide (SCP) and mixture (MIX).

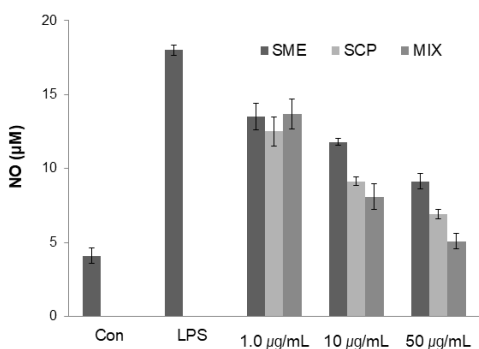


Fig. 2. Nitric oxide inhibitory activities of sea mustard extract (SME), starfish collagen peptide (SCP) and mixture (MIX).

3.3 Tyrosinase inhibitory activity

The tyrosinase inhibitory activity of SME, SCP and MIX is shown in Fig. 3. When treated with 1.0, 10 and 50 µg/mL of SME, SCP and MIX, tyrosinase inhibitory activity was dose-dependent ($p < 0.05$). The results of treatment with 50 µg/mL of extract showed that tyrosinase inhibition activity of 175% was enhanced when SME was treated at 50 µg/mL compared with 1.0 µg/mL. When treated with 50 µg/mL of SCP, tyrosinase inhibitory activity was enhanced by 148% compared to 1.0 µg/mL of SCP. Tyrosinase inhibitory activity was increased by 218% with MIX treatment.

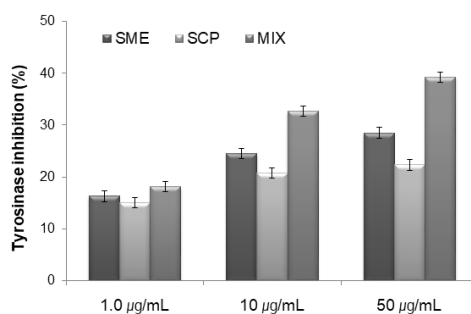


Fig. 3. Tyrosinase inhibitory activities of sea mustard extract (SME), starfish collagen peptide (SCP) and mixture (MIX).

3.4 Elastase inhibitory activity

In order to evaluate the skin wrinkle-improving activity of extracts, various concentrations of SME, SCP and MIX were treated with B16-F10 melanocytes and elastase inhibitory activities were analysed (Fig. 4). The elastase inhibitory activity was increased in a dose-dependent manner for the extract ($p < 0.05$). Especially, 50 µg/mL of MIX treatment resulted in an increased elastase inhibitory activity of 212–268% and 176–207%, respectively, compared to SME or SCP alone.

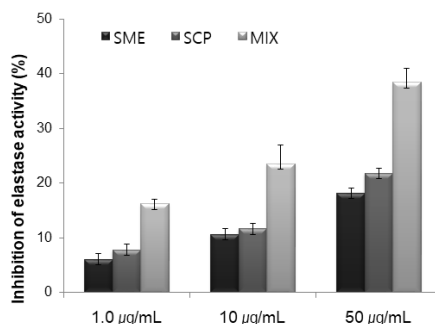


Fig. 4. Elastase inhibitory activities of sea mustard extract (SME), starfish collagen peptide (SCP) and mixture (MIX).

4. Discussion

Over the past few decades, the cosmetics industry has grown to a global scale. The focus of

attention recently has been the development and application of natural additives derived from marine organisms [24]. The main materials for the development of marine biologically derived cosmetic additives are marine algae, microalgae, and bacteria. The use of collagen peptides derived from various marine animals has been increasing in recent years [25]. The development of skin care-related materials such as moisturisers, preventors of skin aging, and topical photoprotectors has been studied mainly for marine biologically active cosmetic additives [24]. The activity of cosmetic ingredients related to skin care can be assessed in a variety of ways, but anti-inflammatory, whitening, and skin wrinkle-improving activity evaluation methods are widely used [11,12,15].

Inflammation is a local phenomenon for defending against foreign body infections, intrusions, and physiochemical damage, but excess biopsy responses cause inflammatory diseases by damaging normal tissues around inflammation sites [19,20]. A large amount of nitric oxide (NO) is produced during the inflammatory reaction. NO plays an important role in killing bacteria and eliminating tumours, but excessive NO production in pathological states causes inflammation, leading to tissue damage, gene mutation and nerve damage [26,27]. Whitening is a function that helps the skin by preventing the deposition of excessive melanin pigment in the skin, or by thinning the colour of the melanin pigment previously deposited and inhibiting the formation of melasma or freckles [28,29]. Melanin pigments in human skin are a major mechanism to protect against UV damage, but they cause undesirable problems such as melasma, freckles, senile lentigines or abnormal pigmentation. Because tyrosinase is involved in the melanin biosynthesis in human skin, tyrosinase inhibitors are known to be important ingredients in cosmetics for their whitening effects [21]. Wrinkle improvement

involves maintaining or enhancing elasticity of the skin. In this regard, elastase activity is known to increase skin aging and reduce skin elasticity [18,23]. Therefore, inhibitors of elastin-degrading enzymes can be used for inhibiting skin aging and improving wrinkles [15].

In this study, we evaluated the anti-inflammatory, whitening and wrinkle-reducing activities of SME, SCP and MIX. SME and SCP alone showed inhibition of NO production (Fig. 2), tyrosinase activity (Fig. 3) and elastase inhibition activity (Fig. 4), but a synergistic effect was observed when MIX was used. These results suggest that it may be better to mix two or more extracts than to use a single extract as a cosmetic additive.

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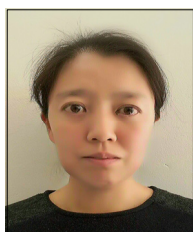
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⟨Research Interests⟩

Marine Biotechnology, Cosmetic

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