

Convergence Study on Preparation of Anti-aging Peptides from Fish Collagen Hydrolysates

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콜라겐 단백가수물을 이용한 항노화 펩타이드 제조에 대한 융합 연구

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Abstract An anti-aging peptide was prepared from fish collagen hydrolysate (FCH) by ultrafiltration (MWCO; 1 kDa) and reversed-phased high performance liquid chromatography (RP-HPLC). Its anti-aging properties were evaluated based on the procollagen-synthesizing and MMP-1-inhibiting activities in Hs68 cells. A potent anti-aging peptide (fraction I-I) increased collagen synthesis by 46% and also inhibited MMP-1 secretion by 77%, compared with unpurified FCH. The amino acid sequence of fraction I-I was identified to be Gly-Arg-Arg-Gly-Asn-Lys (GRRGNK; the repeating Gly-X-Y sequence in collagen), and it had a molecular mass of 686.175 Da. It revealed that the anti-aging activity of GRRGNK was mainly due to skin protective effects. These results demonstrated that fish collagen hydrolysate may be a potential source of anti-aging peptides, which could be utilized in various field, including foods, cosmetics, and pharmaceuticals.

Key Words : Fish collagen hydrolysate, Anti-aging peptide, Collagen synthesis, MMP-1 inhibition, GRRGNK

요약 본 연구에서는 어류 유래 콜라겐 가수분해물로부터 한외여과법(MWCO; 1 kDa)과 역상액체크로마토그래피를 이용하여 항노화 활성 기능성 펩타이드를 분리, 정제하고자 하였다. 펩타이드의 분리, 정제에 따른 항노화 활성 변화는 Hs68 세포를 사용하여 procollagen 합성능과 MMP-1 저해능을 측정하여 확인하였다. 콜라겐 가수분해물과 비교하여 최종 분리, 정제된 기능성 펩타이드의 procollagen 합성과 MMP-1 저해는 각각 46%와 77% 향상되었다. 또한 기능성 펩타이드의 구조는 Gly-Arg-Arg-Gly-Asn-Lys (GRRGNK; 콜라겐의 기본 구조인 Gly-X-Y sequence와 유사)의 서열을 보였고, 분자량은 686.175 Da으로 분석되었다. 따라서 본 연구에서는 어류 콜라겐 가수분해물로부터 분리한 펩타이드가 식품, 화장품, 의약품 등의 피부노화 지연 효과를 보이는 기능성 원료로 다양하게 사용할 수 있는 가능성을 확인하였다.

주제어 : 어류 콜라겐 가수분해물, 항노화 펩타이드, GRRGNK, 콜라겐 합성, MMP-1 저해

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1. Introduction

Collagen, which makes up nearly 80% of the dermis, is categorized into two types (Types 1 and 3). Type 1 collagen is a major constituent of the dermis, whereas there is a limited quantity of type 3 collagen. Type 1 and type 3 collagens are precursor molecules, which are referred to as procollagen, and are synthesized by dermal fibroblasts [1]. Collagen develops a right-handed triple helical structure and sustains skin elasticity [2].

Skin aging, which is associated with wrinkle formation, is caused by imbalance between the synthesis and breakdown of collagen. The enzyme matrix metalloproteinase-1 (MMP-1) destroys collagen (Types 1 and 3) in the dermis [3]. MMP-1 production is facilitated for two reasons, the natural aging process and exposure to UV radiation, and is a critical element in skin aging [4]. It is demonstrated that the quantity of procollagen is reduced in photo-aged skin compared with that of normal skin [5].

Anti-aging properties of collagen have been the main concern of various fields, such as food, cosmetics, and pharmaceuticals [6]. Until recently, the majority of collagen utilized has been extracted from cows and pigs. However, the intake of cow's collagen has been associated with the fear of diseases, such as bovine spongiform encephalopathy (BSE), and the intake of pig's collagen has been severely restricted due to religious practices [7]. Therefore, by-products, such as scales and skin generated by the processing of marine products, could be an alternative source of collagen. Recent studies have reported that collagen hydrolysates or peptides isolated from jellyfish [8], fish scales [9], and *Rhopilema esculentum* skin [10], showed anti-aging effects with increased collagen synthesis and reduced MMP-1 secretion.

Although there have been several researches describing the anti-aging effects of collagen

hydrolysates, only a few peptides have been identified. Furthermore, collagen peptides from different sources, parts, and food each have distinct amino acid contents, which relate to their anti-aging activities [11]. Therefore, the aim of this study was to isolate and identify an anti-aging peptide from fish collagen hydrolysates (FCHs). Also, we investigated the relation between distinct amino acid contents and anti-aging activities of the anti-aging peptide from FCHs. To this end, potential anti-aging peptides were isolated using high-performance liquid chromatography (HPLC), and procollagen synthesis and MMP-1 production were analyzed to characterize the anti-aging ability of the separated fractions.

2. Materials and Methods

2.1 Materials

FCHs, produced from tilapia scales hydrolyzed by a neutral protease (*Bacillus amyloliquefaciens*), were obtained from Nong Shim Co., Ltd (Seoul, Korea). The molecular mass of FCH had an average of 2 kDa, and its amino acid composition is presented in Table 1 [12].

Hs68 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Formic acid and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich Chemical Co. Ltd. (St. Louis, MO, USA). Phosphate-buffered saline (PBS) and penicillin-streptomycin were produced by Lonza (Walkersville, MD, USA). Fetal bovine serum (FBS) and 0.25% trypsin-EDTA Dulbecco's modified Eagle medium (DMEM) were purchased from Gibco Life Technologies (Grand Island, NY, USA). Acetonitrile and water for HPLC were produced by J.T. Baker (Phillipsburg, NJ, USA).

Table 1. Amino acid composition of fish collagen hydrolysate (FCH) (Data citation in the reference of 12)

Amino acid	Composition (g/100 g) ^a
Glycine	23.60
Alanine	11.78
Proline	11.01
Glutamic acid + glutamine	10.16
Hydroxyproline	8.92
Arginine	7.91
Aspartic acid + asparagine	5.59
Serine	3.54
Threonine	3.28
Lysine	3.19
Leucine	2.85
Valine	2.36
Phenylalanine	1.73
Isoleucine	1.40
Histidine	1.10

^a Expressed as grams per 100 g of amino acids.

2.2 Cytotoxicity of FCHs

Hs68 cells were cultivated in DMEM supplemented with 10% FBS and 5% penicillin-streptomycin. The cells were incubated in an atmosphere of 5% CO₂ at 37°C. To evaluate the cytotoxicity of FCHs, cell metabolic activity was measured by the MTT method that is based on the diversion of yellow MTT to purple formazan when the living cells metabolize [13]. A total of 180 µL of cells were added in a 96-well plate at a concentration of 1×10^4 cells/well and incubated for 24 hours at 37°C and 5% CO₂. After appropriate incubation time, cells were treated by FCHs at various concentrations and incubated for 24 hours. After cultivation, 20 µL of MTT (0.5 mg/mL in PBS) was added to the well and incubated for 4 hours. After incubation, the plate was centrifuged at 1,500 rpm for 5 min to deposit the formed formazan crystals and the supernatant was discarded. Next, to solubilize the formazan salts, 150 µL of dimethylsulfoxide (DMSO) was added. The absorbance of the formazan salts in each well was measured at 540 nm in a microplatereader (iMark Microplate Reader,

BioRad, Hercules, CA, USA).

2.3 Procollagen synthesis assay

Hs68 cells were added in a 48-well plate at a concentration of 5×10^4 cells/well and cultivated at 37°C for 24 hours. After removing the supernatant of each well, the cells were rinsed with PBS solution. All samples for testing were prepared in serum-free DMEM and added to the cells. After cultivation for 24 hours, the supernatant was gathered to determine the quantity of procollagen using a procollagen type I C-peptide assay kit (Takara Bio, Japan).

2.4 UV-induced collagenase inhibition assay

Hs68 cells were added in to a 48-well plate at a concentration of 5×10^4 cells/well and cultivated at 37°C for 24 hours. After removing the supernatant of each well, the cells were rinsed with PBS. All samples for testing were prepared in serum-free DMEM and were added to the cells. After 6 hours of cultivation, the cells were rinsed with PBS solution two times. Each well covered with 100 µL of PBS was exposed to 10 J/cm² UV A with a UVT UV lamp (Dongseo Science Co., Ltd, Korea). The PBS was removed, after which the serum-free medium was added to the cell. The cells were incubated for 24 hours, and the supernatant was gathered for determining the quantity of collagenase, which was measured using an MMP-1 Human Biotrak ELISA kit (GE Healthcare Life Science, Piscataway, NJ, USA).

2.5 Ultrafiltration

Fractionation of FCHs was performed by an Amicon model 8400 UF unit (Millipore Corp., Billerica, MA) using a Millipore membrane with a molecular weight cut-off of 1 kDa. Two fractions with MW <1 kDa and >1 kD were obtained. The fraction with the highest anti-aging activity was further separated by reversed-phased high performance liquid chromatography.

2.6 Reversed-phased high performance liquid chromatography (RP-HPLC)

The FCH fraction with low molecular weight (<1 kDa) was separated by RP-HPLC on a Zorbax Eclipse XDB-C₁₈ column (9.4x250 mm², 5 μ m, Agilent Technologies, Palo Alto, CA, USA). The sample was eluted with 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) using 5% A at 0-5 min, 95% A at 10 min, and 5% A at 15 min at a flow rate of 3.0 mL/min. The eluted peaks were monitored by a UV detector at 280 nm. The fraction showing the highest anti-aging activity was collected and concentrated using a rotary evaporator (Eyela N-1000, Tokyo Rikakikai Co., Ltd.). This fraction was then resolved on a Zorbax C₁₈ column (4.6x150 mm, 5 μ m, Agilent Technologies, Palo Alto, CA, USA) at a flow rate of 1 mL/min. A mixture of 0.1% formic acid in water and 0.1% formic acid in acetonitrile (95:5 v/v) was used as the mobile phase. For each separation, the peptide peaks were monitored at 280 nm. The purification procedures were repeated until the samples were purified to a single peak.

2.7 Amino acid composition

Amino acid was analyzed using a Waters HPLC system equipped with a Waters 510 pump and a Waters Pico-Tag column (3.9 x 300 mm, 4 μ m). The mobile phases consisted of 140 mM sodium acetate in 6% acetonitrile (A) and 60% acetonitrile (B), and the following gradient was used: 86% A at 9 min, 80% A at 9.2 min, 54% A at 17.5 min, and 0% A at 17.7 min. The sample was hydrolyzed with 6N HCl and labeled with phenylisothiocyanate. After labeling, 10 μ L of the sample was injected into the column, and peaks were monitored at 250 nm by a UV detector.

2.8 Molecular mass and peptide sequencing

The intact mass of the purified peptide was determined by LC/MS analysis using an Agilent 1260 Infinity LC system coupled with an Agilent 6530 Accurate-Mass Q-TOF instrument. The

sample (1 μ L, 10 mg/mL) was loaded on a Zorbax Eclipse Plus C₁₈ (2.1 x 50 mm, 1.8 μ m) column (Agilent Technologies, Palo Alto, CA, USA). The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B), and the following gradient was used: 95% A at 0-5 min, 5% A at 10 min, and 95% A at 15 min. Peptides were detected by full scan mass analysis over the range 0-2,000 (m/z). For peptide sequence analysis, Edman sequencing was performed by standard procedures using an Applied Biosystems Procise Sequencer (Applied Biosystems, Couatoboeuf, France).

2.9 Synthesis of the peptide

The peptide, Gly-Arg-Arg-Gly-Asn-Lys (GRRGNK), was synthesized at AnyGen Ltd (Gwangju, Republic of Korea). The purity of the synthesized peptide was more than 95% as measured by HPLC. The anti-aging properties of the synthesized peptide were evaluated.

2.10 Statistical analysis

SPSS (SPSS Version 21.0, SPSS Inc., Chicago, IL, USA) was used for statistical examination. Data were analyzed by one-way analysis of variance (ANOVA) with Duncan's test ($p < 0.05$). All experiments were conducted at least in triplicate, and quantitative results are indicated as the mean \pm standard deviation (SD).

3. Results and Discussion

3.1 Cytotoxicity and anti-aging properties of FCH

The cytotoxicity of FCHs to Hs68 cells was assessed by the MTT assay. As can be seen in Fig. 1 [12], the metabolic activity of Hs68 cells treated with FCHs (0-1,000 μ g/mL) was on average more than 80% of that of the control; FCHs had no toxic effect on the Hs68 cells for any of the tested concentrations.

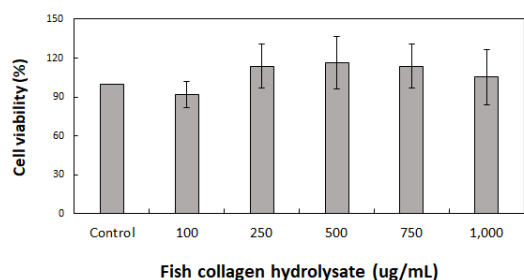


Fig. 1. Cytotoxicity of Hs68 cells treated with FCHs (Data citation in the reference of 12).

3.2 Anti-aging properties of the fractions from FCH

The quantities of procollagen and MMP-1 in Hs68 cells treated with ultrafiltration fractions (<1 kDa, >1 kDa) were evaluated by a procollagen type I C-peptide ELISA assay. As exhibited in Fig. 2(A), type 1 procollagen synthesis was increased in all cells treated with the three types of samples. The procollagen formation of these

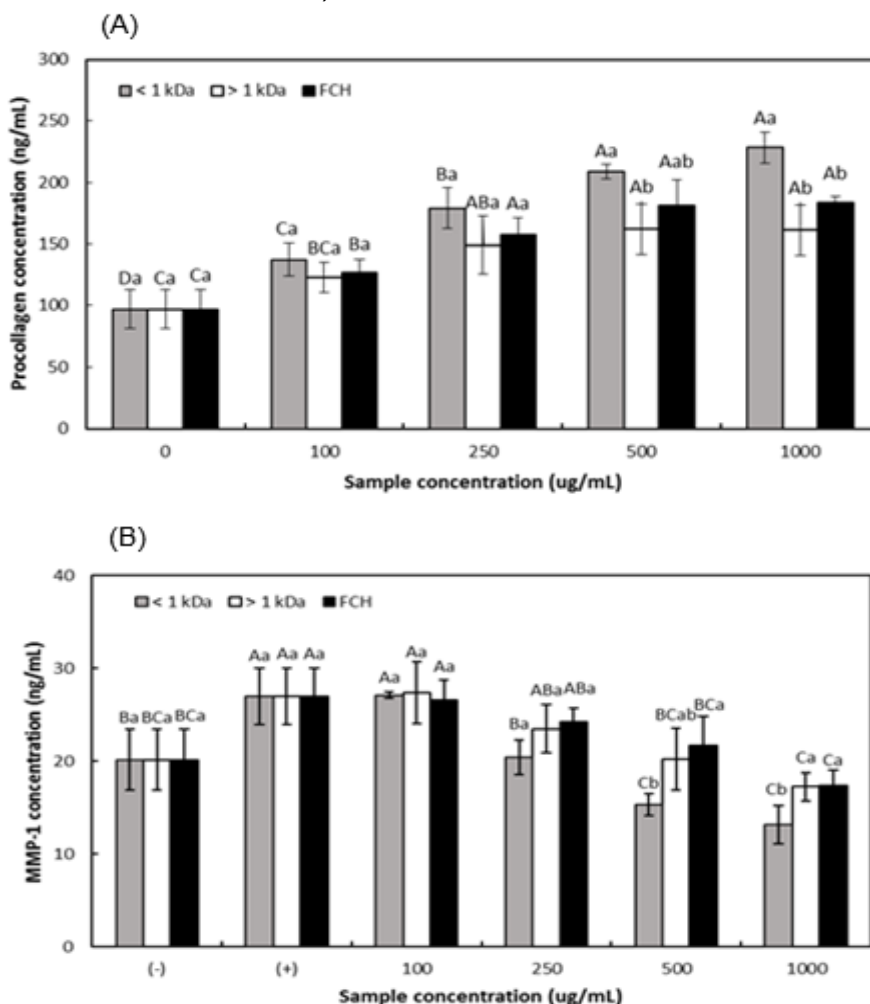


Fig. 2. Procollagen synthesis by Hs68 cells treated with FCHs and two fractions (<1 kDa, >1 kDa) obtained from ultrafiltration. (A) Quantitation of MMP-1 production by Hs68 cells treated with fish collagen hydrolysates (FCHs), their <1 kDa fraction, and their >1 kDa fraction for 24 hrs after UVA irradiation (10 J/cm²). (B) (-) and (+) designate the two control groups (unexposed and exposed to UVA, respectively). Means with capital letters for the same sample and small letters for the same concentration are significantly different ($p < 0.05$).

samples might be related to the amino acid composition of FCH (Table 1). The six major amino acids, Gly, Ala, Pro, Glu, Hyp, and Arg, accounted for nearly 73.4% of FCHs. Ala has been found to relate to collagen synthesis [14], and Pro-Hyp functions as a fibroblast growth factor, which facilitates collagen formation [15]. Additionally, Arg is a precursor of proline, which contributes to collagen synthesis [16].

In particular, the <1 kDa fraction significantly increased the amount of synthesized type 1 collagen in a dose-dependent manner at all tested concentrations (100, 250, 500, and 1000 $\mu\text{g/mL}$; 137.01 ng/mL, 179.18 ng/mL, 208.76 ng/mL, and 228.4 ng/mL), while FCHs and >1 kDa fraction did not show this dose-dependent activity. Furthermore, at the concentrations of 500 and 1,000 $\mu\text{g/mL}$, the <1 kDa fraction produced more than twice the amount of collagen synthesis compared to that of the control (97.02 ng/mL) and exhibited a significant difference in the production of collagen compared with FCHs and >1 kDa fraction ($p < 0.05$).

The amounts of UV-induced MMP-1 in the Hs68 cells treated with FCHs and ultrafiltration fractions (<1 kDa, >1 kDa) are exhibited in Fig. 2(B). All three samples showed a dose-dependent inhibition of MMP-1. At the concentrations of 500-1,000 $\mu\text{g/mL}$, all samples significantly reduced the production of MMP-1. Several studies have shown that Gly, Glu, Pro, and Hyp consisting of FCH (Table 1) inhibit MMP secretion [8,16]. The MMP-1 inhibition effect of these fish collagen-derived samples may be due to their amino acid composition.

In particular, the <1 kDa fraction was found to inhibit the MMP-1 in Hs68 cells the most effectively among the three samples. In the concentration range of 250-1,000 $\mu\text{g/mL}$, the <1 kDa fraction significantly inhibited the MMP-1 expression (20.43 ng/mL, 15.33 ng/mL, and 13.18 ng/mL) compared to the UV-irradiated control cells (26.98 ng/mL). Therefore, the <1 kDa

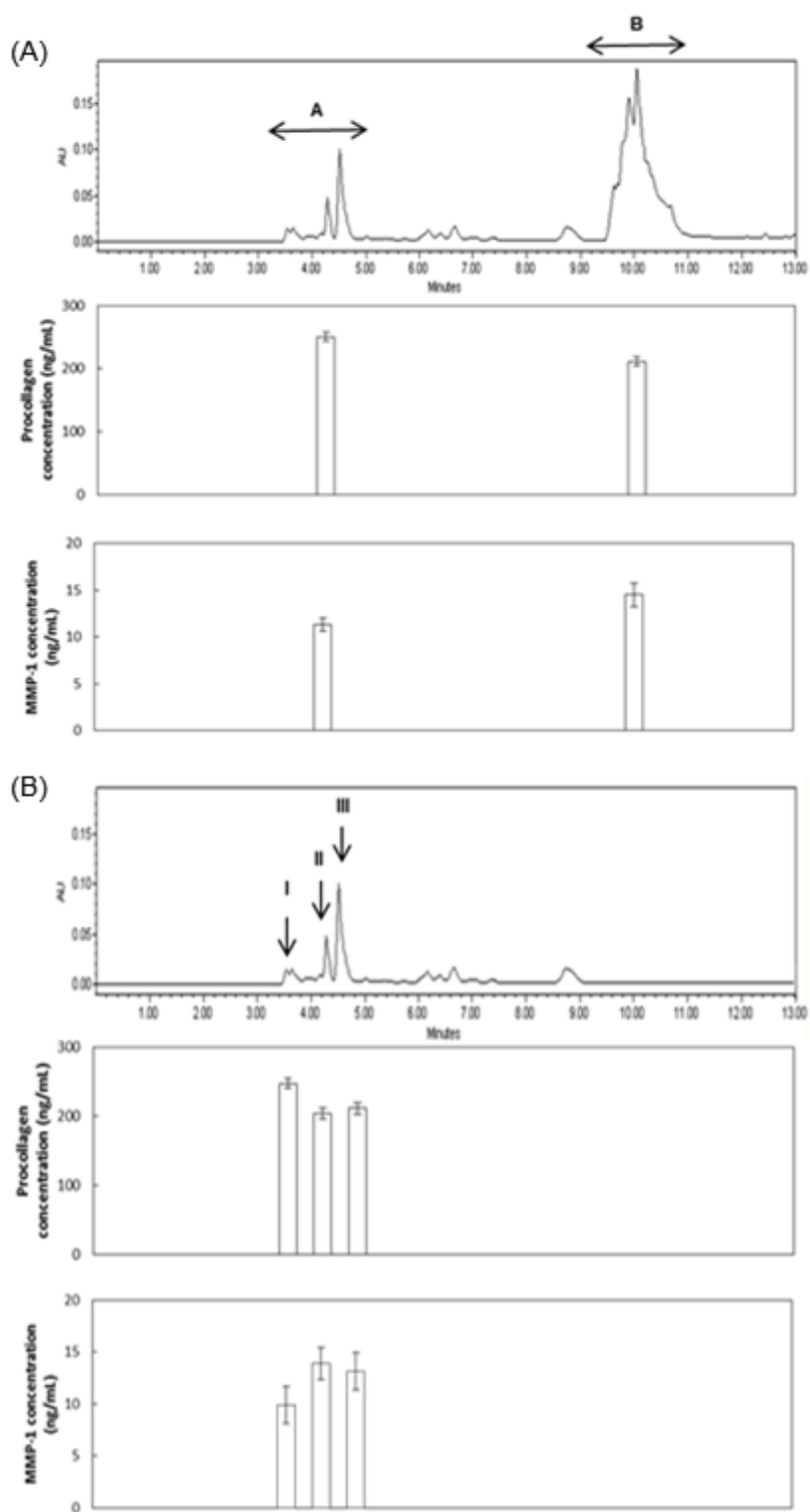
fraction was considered to have the highest anti-aging activity and thus was further isolated into a Zorbax C₁₈ column.

3.3 Anti-aging properties of fractions isolated from HPLC

After ultrafiltration of FCHs, the <1 kDa fraction was separated by HPLC on a C₁₈ semi-prep column, leading to the collection of two fractions (A and B) (Fig. 3(A)). The amounts of procollagen released in fractions A and B were 241.97 ± 5.02 ng/mL and 213.48 ± 3.01 ng/mL, respectively. The collagen-synthesizing and MMP-1-inhibiting abilities of fraction A were 13.35% and 22.15% greater, respectively, compared to those of fraction B. Fraction A was further resolved using a Zorbax analysis C₁₈ column; three fractions (I, II, and III) were collected and lyophilized (Fig. 3(B)). Treatment with fraction I yielded 21.28% higher collagen synthesis and 22.51% lower MMP-1 expression compared with the least bioactive fraction (II). The fraction with the highest anti-aging potential, fraction I, was further resolved using an analysis column. Fig. 3(C) shows the two additional fractions (I-I and I-II) isolated from fraction I; treatment with fraction I-I yielded 52.67% higher procollagen production and 23.21% lower MMP-1 expression than treatment with fraction I-II. Therefore, the most bioactive fraction (I-I) was purified into a clear single peak, as shown in Fig. 3(D).

The procollagen synthesis, MMP-1 inhibition, and the yield of the anti-aging peptides at each separation were exhibited in Table 2.

Fig. 3. Purification scheme for obtaining anti-aging peptides from fish collagen hydrolysates. (A) First isolation of the <1 kDa fraction using a semi-preparative column, (B) second isolation of fraction A using an analytical column, (C) third isolation of fraction I, and (D) final purification of fraction I-I.



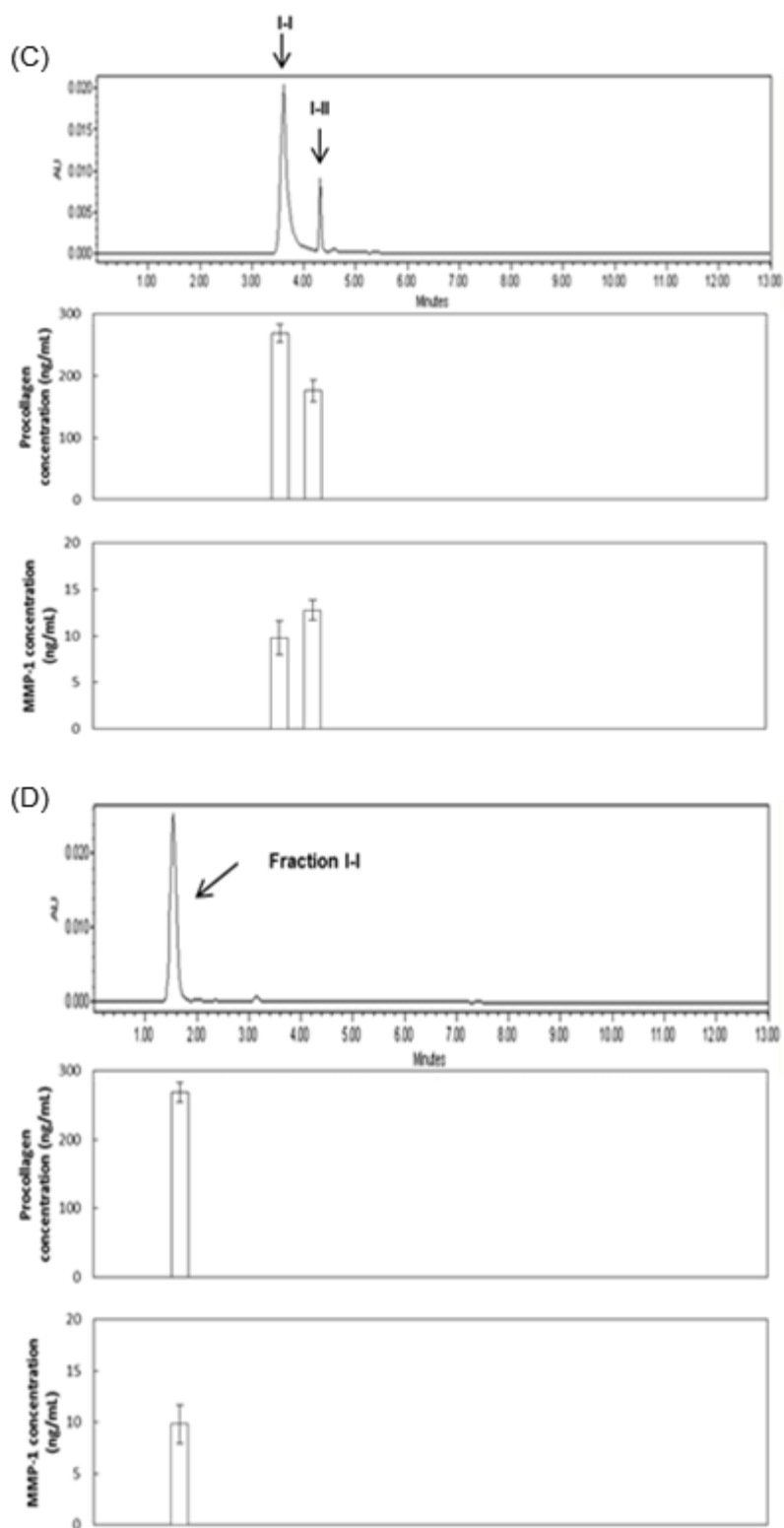


Table 2. Purification of the anti-aging peptide from fish collagen hydrolysate (FCH)

Purification stage ^a	Procollagen (ng/mL)	MMP-1 (ng/mL)	Yield (%)
FCH	183.60 ± 4.93	17.38 ± 1.66	100
Ultrafiltration (<1 kDa)	228.40 ± 12.68	13.18 ± 2.05	40.10 ± 9.12
First RP-HPLC (fraction A)	241.97 ± 5.02	11.30 ± 0.69	15.34 ± 4.04
Second RP-HPLC (fraction I)	247.65 ± 8.26	10.76 ± 0.60	2.23 ± 2.18
Third RP-HPLC (fraction I-I)	268.95 ± 27.20	9.80 ± 1.83	1.40 ± 0.58

^a The concentration of the sample for each purification stage was 1 mg/mL.

3.4 Identification of the anti-aging peptide

The amino acid composition of FCH fraction I-I is presented in Table 3 [12]. The major amino acids in fraction I-I were Gly, Ala, Arg, Lys, Glu; together, these six amino acids comprised approximately 85.15% of fraction I-I. Gly was the most abundant amino acid in fraction I-I, which may be due to the repeating Gly-X-Y sequence in collagen. Additionally, the high Ala and Lys FCH contents are in agreement with previous studies showing that these two amino acids contribute to collagen formation [14]. Arg is a precursor of proline synthesis [16], which is an important amino acid in collagen synthesis. Thus, the amino composition of fraction I-I is in good agreement with the major components of collagen hydrolysates.

Table 3. Amino acid composition of the purified peptide (fraction I-I) from fish collagen hydrolysate (FCH) (Data citation in the reference of 12)

Amino acid	Composition (g/100 g) ^a
Glycine	32.44
Alanine	23.89
Arginine	15.61
Lysine	8.06
Glutamic acid + glutamine	5.15
Threonine	3.53
Aspartic acid + asparagine	1.71
Phenylalanine	1.58
Hydroxyproline	1.16
Histidine	1.09

^a Expressed as grams per 100 g of amino acids.

The molecular mass of fraction I-I was determined using LC/MS. The anti-aging peptide was determined to have a molecular mass of 686.175 Da. Through Edman degradation sequencing, the amino acid sequence of fraction I-I was identified to be Gly-Arg-Arg-Gly-Asn-Lys (GRRGNK).

3.5 Anti-aging properties of the synthesized peptide

In order to confirm the anti-aging activity of the isolated peptide, the peptide (GRRGNK) was synthesized. The anti-aging activity of GRRGNK was determined to identify the active component in fraction I-I (Table 4). No significant difference was observed in the amounts of procollagen synthesis and MMP-1 inhibition stimulated by fraction I-I and GRRGNK. Additionally, GRRGNK yielded 81.8% higher collagen synthesis and 63.57% lower MMP-1 expression compared with the UV-irradiated control. Thus, the purified peptide was verified to be actually relevant to the anti-aging effect.

Table 4. Anti-aging properties of the purified peptide (fraction I-I) and the synthesized peptide (GRRGNK)

Samples ^a	Procollagen (ng/mL)	MMP-1 (ng/mL)
Fraction I-I	268.95 ± 27.20 a	9.80 ± 1.83 b
GRRGNK	267.28 ± 13.75 a	9.50 ± 1.20 b

^a The concentration of the sample was 1 mg/mL.

Means with the same letters within the same column are not significantly different ($p > 0.05$).

4. Conclusion

A bioactive peptide with anti-aging properties was isolated using ultrafiltration and high-performance chromatography from FCHs, and its sequence was identified. Although the anti-aging properties of GRRGNK and its derivatives have not been previously studied, peptides with similar compositions and sequences have been investigated. In one study, Arg-Gly-Asn was reported to show similar effects to Arg-Gly-Asp [17], a bioactive tripeptide related to cell binding and adhesion. Cell binding proteins have also been reported to enhance cell proliferation, since cells express receptors that enable binding to tissue collagen structures [18]. Thus, the Arg-Gly-Asn portion of the isolated GRRGNK peptide may enhance fibroblast proliferation, leading to higher collagen synthesis. It is also of note that Gly-Asn-Lys, which can be located at the C-terminus of the GRRGNK peptide, exhibits a similar structure to Gly-His-Lys (GHK). It is recognized that GHK is a tripeptide involved in wound healing and copper-GHK complexes have been reported to stimulate fibroblast growth and increase collagen production [19,20].

Furthermore, a variety of collagen-derived peptides composed of diverse combinations of amino acids were shown to have collagen synthesizing and MMP-1 inhibiting effects. A research study reported that Chum salmon skin hydrolysate consisting of Gly, Glu, Pro, and Hyp significantly increased collagen production and inhibited MMP-1 expression [21]. Additionally, collagen hydrolysate from bovine hide with high contents of Gly, Glu, Arg, Pro, and Hyp showed increased collagen synthesis and decreased MMP-2 production [8]. Jellyfish collagen hydrolysate rich with Gly, Pro, Glu, Asp, and Ala was reported to show collagen synthesis and antioxidant activity [8]. Another study revealed that specific ratios of Arg, Glu, Val, Ile, and Leu could synthesize collagen and inhibit skin aging

[22]. These studies show that there can be numerous combinations of amino acid compositions that could contribute to skin protective effects. Thus, it can be assumed that the identified GRRGNK peptide possesses a high possibility as a potential anti-aging peptide. These studies indicate that the identified GRRGNK peptide possesses high potential as an anti-aging peptide. Specially, the <1 kDa fraction of FCH exhibited effective intestinal permeation and digestive enzyme stability [12], indicating that it is suitable for the application in anti-aging foods and cosmetic products.

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