J Appl Biol Chem (2020) 63(3), 203–209
https://doi.org/10.3839/jabc.2020.028

Online ISSN 2234-7941
Print ISSN 1976-0442

Article: Food Science

Anti-aging skin and antioxidant assays of protein hydrolysates obtained from salted shrimp fermented with *Salinivibrio cibaria* BAO-01

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Received: 25 June 2020 / Accepted: 12 August 2020 / Published Online: 30 September 2020
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Abstract This study focused on the preparation and characterization of anti-wrinkle peptides from the salted and fermented shrimp (*Acetes japonicus*) inoculated with *Salinivibrio cibaria* BAO-01 (SFSC). The results showed the proximate composition of SFSC to be 9.23% water, 75.32% protein, 0.23% fat, and 13.3 mg/g ash. Interestingly, the *S. cibaria* fermentation significantly increased the amount of methionine, leucine, and arginine. The *in vitro* antioxidant activity was assayed by the diphenylpicrylhydrazyl method and its IC_{50} value was found to be 43.02±2.84 μg/mL. It was observed to inhibit the activity of elastase, tyrosinase, collagenase, and hyaluronidase. The IC_{50} values of SFSC were 182.75±12.38 μg/mL for anti-elastase activity, 186.78±7.95 μg/mL for anti-tyrosinase activity, 444.4±34.81 μg/mL for anti-collagenase activity, and 1447.95±28.92 μg/mL for anti-hyaluronidase activity. These results suggest that salted and fermented shrimp has strong potential for the development of nutricosmetic products.

Keywords Anti-aging · Antioxidant · Protein · Salted shrimp

Introduction

The presence of wrinkles is an indication of the aging of skin, an inevitable process instigated by several factors, both intrinsic and extrinsic [1]. Intrinsic aging of skin, also called age-dependent aging or chronologic aging, is caused by internal physiological factors, while extrinsic aging is caused by many external factors, including exposure of the skin to UV radiation, cigarette smoking, and air pollution. These factors generate exogenous reactive oxygen species, which overwhelm antioxidant defense mechanisms, leading to degradation of elastin and collagen, deposition of glycosaminoglycans, and induction of melanin biosynthesis [2]. Exposure to UV increases the production of collagenase, elastase, and hyaluronidase, which degrade the abundant collagen and elastin of the extracellular matrix (ECM) [3]. Collagenase, a zinc-dependent endopeptidase, is capable of degrading type 1 collagen. Elastase, a protease enzyme, breaks down elastin and collagen which determine the mechanical and structural properties of the connective tissue in the ECM. Hyaluronidase is a mucopolysaccharide-degrading enzyme that hydrolyses the β-1,4-glycosidic bonds of hyaluronic acid and induces a decrease in its viscosity. Inhibitors of these enzymes can be used for the process of renewal and recovery of aging skin.

Anti-aging strategies for the skin are complex, and can involve multiple, concurrent treatments. These include cosmetic care, topical bioactive agents, invasive procedures, systemic agents, preventative medicine, the avoidance of exogenous factors and correction of lifestyle and habits [4]. Nutrition is a promising strategy for cellular maintenance and renewal, and for erasing the effects of skin aging, that is also healthy and safe. Peptide molecules, especially small fragments (2-20 amino acids), have been investigated as a safe, emerging therapy for the care of skin, nails, and hair [5,6]. Recently, the skin-care industry has begun to manufacture peptide-based products, including creams, lotions, and nutritional supplements [6]. There is a current need for studies to evaluate potential peptide sources for nutricosmetics, and to establish their effects on human health and on metabolic parameters.

Fermented fish products are a rich source of bioactive peptides. During the fermentation process, cleavage of food proteins by microbial or indigenous proteases yields bioactive peptides, leading to a substantial increase in the biological activity of the

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food [7]. Over the last decade, many peptides have been identified in fermented fish foods and have been shown to exhibit activities such as antihypertensive, antimicrobial, obesity control, immunomodulatory, anticancer, antithrombotic, opioid, and antioxidant [8, 10]. Some cosmeceutical products containing fish protein and peptides can enhance collagen synthesis, chemotaxis, and protect skin from the effects of UV radiation [11]. The anti-photoaging effect of a low-molecular-weight collagen peptide purified from catfish skin (*Pangasius hypophthalmus*) was evaluated in animal models and in a clinical trial [12].

Salted and fermented shrimp products are popular in South-East Asian countries, such as Korea, Thailand, China, and Japan [13]. In Korea, salted and fermented shrimps are produced by mixing whole shrimp, *Acetes japonicus* with 5-20% salt and storing for several months at ambient temperature. The fish fermentation process consists of the transformation of organic substances into simpler compounds, such as amino acids, peptides, and various nitrogenous compounds. There are some studies on the antioxidant activity of shrimp peptides; however, to the best of our knowledge, no report exists of their anti-wrinkle properties. *Salinivibrio* is the predominant bacterial genus in fermented seafoods [14]. These bacteria produce numerous enzymes such as lipase, cellulase, and chitinase with antifungal and antimicrobial properties [15]. Therefore, the aim of this study was to determine the anti-elastase, anti-collagenase, and anti-hyaluronidase activities of salted and fermented shrimp protein extracts, obtained from salted shrimp fermented with *Salinivibrio cibaria*.

Materials and Methods

Salted and fermented shrimp preparation

The use of *Salinivibrio cibaria* BAO-01 as a starter culture was previous characterized [16]. A 1% (v/v) inoculum is placed in marine broth (Difco, Detroit, MI, USA), supplemented with 5% sodium chloride, and incubated at 37 °C for 24 h in static conditions. To prepare salted and fermented shrimp inoculated with *Salinivibrio cibaria* BAO-01 (SFSC), fresh shrimp (*Acetes japonicus*) was provided by Shinan Saujeot Inc., mixed well with solar salt (sun-evaporated) at a ratio of 20% salt to 80% shrimp, and stacked in glass containers. A 1% starter culture (10^8 cells/mL) was used and the containers were capped and allowed to stand at room temperature (25 °C) for 3 min. The containers contain shrimps and salts without starter culture as used as control (SFS). After the fermentation process was complete, SFS and SFSC samples were minced with a heavy-duty meat mincer (MGT0012, Nemco Food Equipment Ltd., Hicksville, OH, USA), freeze-dried and stored at −20 °C.

Preparation of water extract

The salted and fermented shrimp protein extract was prepared using an enzymatic process. Fifty grams of thawed sample mince was suspended (1:1, w/v) in distilled water in Sorvall™ bottles and the mixture was brought to 90 °C for 30 min to inactivate the endogenous enzymes. The mixture was then homogenized, and pH was adjusted to 8.5 with 1 N NaOH. The enzyme alcalase®, a bacterial endoproteinase from *Bacillus licheniformis,* with a declared activity of 2.4 AU/g and a density of 1.18 g/mL, was procured from Sigma Aldrich (St. Louis, MO, USA) and added at an enzyme to substrate ratio of 1.5 g/100 g substrate. The enzymatic process was carried out at 60 °C for 20 min in a shaking water bath. After the completion of hydrolysis, alcalase was deactivated by holding the sample at 90 °C for 5 min. After cooling to room temperature, the hydrolyzed mixture was filtered with sterile gauze, and centrifuged (AvantiTM J-25 I, Beckman Coulter, California, USA) at 7500×g for 15 min at 4 °C. The supernatant was concentrated in a rotary evaporator, lyophilized using a freeze drier (Freeze dryer, Ilshin FD5505, Siheung, Korea), and stored in polypropylene tubes (50 mL) at −20 °C until use.

Proximate analysis

The extracts was analyzed for moisture, ash, protein, and fat content, according to AOAC official methods [17]. In brief, the samples (1 g) were dried at 105 °C in an oven, to a constant weight. The moisture content was calculated by weight difference before and after the drying process. After that, ash content was determined by incineration of a dried sample mass at 600 °C. The protein content was measured by the Kjeldahl method and quantified by multiplying total percentage nitrogen by a factor of 6.38. The fat content was extracted by chloroform-methanol followed by the Mojonnier method.

Free amino acid composition

To determine the free amino acid composition, samples will firstly mixed with 6% (v/v) perchloric acid and then followed the extraction protocol of Thuy, Okazaki et al. [18]. The lysates were subsequently neutralized and filtered. The filtrate was used for amino acid analysis using the Prominence HPLC System (Shimadzu, Kyoto, Japan) equipped with a column (Shim-pack Amino-Li, 100 mm×6.0 mm i.d.; column temperature, 39 °C; Shimadzu) and pre-column (Shim-pack ISC-30/S0504 Li, 150 mm×4.0 mm i.d.; Shimadzu). Amino acids were detected using a fluorescence detector (RF-10AXL; Shimadzu). The content was reported as mg/g dry weight of sample.

Antioxidant scavenging activity assay

The diphenylpicrylhydrazyl (DPPH)· radical-scavenging activity was determined as described by Yen and Wu [19] using butylated hydroxyanisole (BHA) as the standard compound. The percentage radical scavenging activity was calculated based on the following equation:

$$\text{DPPH Scavenged (\%)} = (A_{cont} - A_{sample})/A_{cont} \times 100$$

A_{cont} and A_{sample} are the absorbance values for the control and sample, respectively.

Elastase inhibitory assay

The elastase inhibitory activity of salted shrimp samples were evaluated *in vitro* using a porcine pancreatic elastase (PPE) enzyme inhibitory assay [20]. Briefly, porcine pancreatic elastase was dissolved in sterile water to make a 100 unit stock solution. The substrate N-Succinyl-Ala-Ala-Ala-p-nitroanilide (AAAPVN) was dissolved in 0.2 M Tris-HCL buffer (pH 8.0) buffer. The extracts were incubated with the enzyme for 25 min before adding substrate to start the reaction. The final reaction mixture (250 μL) contained buffer, 10 μg/mL AAAPVN, 0.001 units PPE and 500 μg/mL of shrimp extract. After incubation for 10 min, the absorbance was measured at 410 nm and inhibition values for each sample were calculated as follows:

$$\text{Inhibition (\%)} = [1 - (\text{Sample} - \text{Blank})/(\text{Control} - \text{Blank})] \times 100$$

where Blank is before incubation, Control is without sample, after incubation. Ursolic acid was used as positive control.

Tyrosinase inhibitory assay

The activity of mushroom tyrosinase was determined spectrophotometrically, as described by Schurink, van Berkel et al. [21]. The reaction mixture (1000 μL) contained 685 μL of 0.1 M phosphate buffer pH 6.5, 15 μL of 2500 U/mL mushroom tyrosinase in phosphate buffer, 200 μL of various concentrations of SFSC extract and 100 μL of 5 mM L-3,4-dihydroxyphenylalanine (DOPA). Once the DOPA was added, the reaction was monitored at 492 nm for dopachrome formation in the mixture. Ursolic acid was used as a positive control. After incubating the mixture at 37 °C for 30 min, the optical density was measured at 490 nm, using a microplate reader.

Collagenase inhibitory assay

The collagenase inhibitory assay was performed with an MMP1 Human ELISA kit (Amersham, Little Chalfont, UK), according to the manufacturer's protocol. The 4phenylazobezyloxylcarbonyl ProLeuGlyProArg (the synthetic substrate) was dissolved in reaction buffer at 0.3 mg/mL, and 250 μL was added in the reaction tube with 100 μL of various concentrations of SFSC extract (1000, 500, 250, 125, and 62.5 μg/mL) or epigallocatechin gallate (EGCG) (20 μM). Collagenase was dissolved in the buffer at 0.2 mg/mL and 150 μL of this was added to the reaction tube. After incubation at 25 °C for 20 min, the reaction was stopped by addition of 6% citric acid. The reaction mixture was separated using 1.5 mL of ethyl acetate. The absorbance was measured at 320 nm using a microplate reader.

Hyaluronidase inhibitory assay

Hyaluronidase inhibition was determined by measuring the amount of β-N-acetylglucosamine formed from sodium hyaluronate. A stock of enzyme was prepared by dissolve bovine hyaluronidase (7420 units) in 1 mL of 0.1 M acetate buffer; pH 5.6. The 500 μL of enzyme stock was activated by mixing with 100 μL calcium chloride (0.25 mM) and then incubated in a water bath at 37 °C for 20 min. The reaction was started by the addition of 500 μL of sample serial dilutions. After 20 min, 500 μL of sodium hyaluronate was added (0.5 mg/mL in 0.1 M acetate buffer; pH 5.6). After a 30-min incubation, the reaction was stopped by adding 100 μL of 0.4 M sodium hydroxide. Next, 500 μL acetylacetone was added, and the mixture was placed in boiling water for 30 min to produce a chromogenic reaction. After cooling to 25 °C, 1.0 mL of p-dimethylaminobenzaldehyde solution was added to the reaction mixture for 20 min at 25 °C. The optical density of the reaction mixture was measured at 555 nm using a microplate reader (Infinite 200; TECAN Group Ltd., Männedorf, Switzerland) Three independent assays were performed in triplicate.

Sensory evaluation

A sensory analysis was performed with a group of 20 untrained panelists to evaluate differences between the SFS and SFSC samples, using a 9-point hedonic scale. The panelists were asked to rate samples for softness, fibrousness, sweetness, bitterness, sourness, aroma intensity, aftertaste, and overall preference with 1 representing "dislike extremely" and 9 representing "like extremely" for each sample. Sensory evaluation was conducted in individual booths to prevent rate score bias.

Statistical analysis

All experiments were conducted in triplicate. Statistical analysis was done by using one-way analysis of variance (ANOVA) to determinate significant differences within and between groups, followed by Duncan's multiple range test. These analyses were performed using the SPSS package (Version 22.0) (SPSS Inc., Chicago, IL, USA). Outcomes of sensory evaluation were analyzed using the paired-sample t-test.

Results and Discussion

Previous studies have revealed that peptides obtained from shrimps have a range of bioactivity, such as antihypertensive, antioxidant, antimicrobial, neuroprotection, antihyperglycemic, and anti-aging [9,22,23]. Therefore, we hypothesized that fermented shrimp might be able to enhance health without having side effects.

Proximate composition

The proximate composition of SFSC extract comprised 9.23% water, 75.32% protein, 0.23% fat, and 13.3 mg/g ash (Table 1). Its high protein content was due to solubilization of protein during enzymatic hydrolysis and the exclusion of insoluble, indigestible, non-protein isolates. This was comparable with the composition of shrimp protein extract, reported in previous studies [24,25]. The moisture content is linked to the type of sample and to the temperatures employed during the drying process. The ash content is in the previously reported range of 8.25-23.4% [25,26]. Variation

Table 1 Proximate composition of salted fermented shrimps

Composition (%)	Moisture	Protein	Fat	Ash
SFS	9.23±1.4	75.32±0.82	0.23±0.08	13.3±0.64
SFSC	9.87±0.6	73.95±0.49	0.34±0.07	14.4±0.38

Values was expressed as g per 100 g dry weight. Mean ± SD from triplicate determinations

Table 2 Amino acid composition of salted and fermented shrimps (mg/g protein)*

Free amino acid	SFS	SFSC
Thr	4.23±0.31	4.12±0.16
Asp	2.83±0.16[b]	3.75±0.21[a]
Ser	3.96±0.15	3.59±0.15
Glu	15.23±0.21	14.93±0.16
Pro	2.13±0.07	2.32±0.13
Gly	2.45±0.12	2.41±0.01
Ala	8.01±0.09	8.03±0.09
Val	6.89±0.11	5.68±0.14
Cys	2.18±0.03	2.35±0.03
Met	4.82±0.12[a]	1.96±0.11[b]
Ile	4.23±0.04	4.23±0.04
Leu	12.42±0.03[a]	9.11±0.06[b]
Tyr	4.06±0.11	4.35±0.05
Phe	4.11±0.12[b]	5.24±0.03[a]
Lys	7.89±0.21[b]	11.84±0.16[a]
His	3.89±0.10	3.59±0.04
Trp	1.23±0.08[b]	4.24±0.04[a]
Arg	9.36±0.12[a]	6.31±0.18[b]
Total essential amino acid (%)	61.9±0.14[a]	56.43±0.09[b]
Total hydrophobic amino acid (%)	43.84±0.13[a]	40.81±0.15[b]
Total hydrophilic amino acid (%)	34.94±0.09	35.5±0.07

*Values presented are means of triplicates ± SD
[a,b]The different letters in the same line represent that there are significant differences at the level of $p < 0.05$

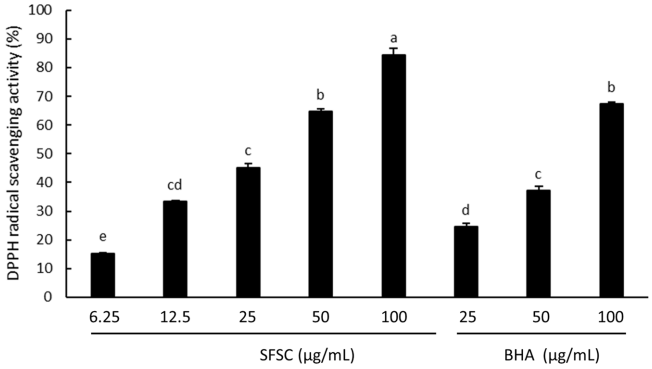

Fig. 1 DPPH· scavenging activities of salted and fermented shrimps. All data are presented as the mean ± SD of triplicate results. [a-e]Values with different letters indicated significant differences in the same free radical at different concentrations ($p < 0.05$)

in ash content is due to the mineral content of the sample and the addition of an acid or base to adjust the pH during hydrolysis [27]. The protein content is higher than the 46.52-47.01% reported by Dhanabalan, Xavier et al. [24] for *Acetes indicus* and Ghorbel-Bellaaj, Maalej et al. [25] from the fermented shrimp waste hydrolysate of *Metapeneaus Monoceros*. It is lower, however, than the 91.37 % reported by Najafian and Babji [8] for fermented Loma fish (*Thynnichthys thynnoides*).

Amino acid composition of protein extract

In order to identify the contribution of *S. cibaria* BAO-01 to the amino acid profile, the comparison between the free amino acid composition of SFS (fermented shrimp paste without *S. cibaria*, the control) and SFSC protein extracts is presented in Table 2. The inoculum of *S. cibaria* significantly increased the total essential amino acid content of the protein extract. SFSC showed a higher proportion of lysine (118.4 mg/g protein) and leucine (91.1 mg/g protein), among essential amino acids, while glutamate (149.3 mg/g protein) contributed the highest proportion of the total amino acid content. Glutamate, which has a savory flavor, was the most abundant amino acid in both SFS and SFSC, in agreement with the composition of other shrimp pastes [24]. The leucine concentration of SFSC was significantly higher than that produced in SFS. Le Vo, Pham et al. [28] produced fresh *A. japonicus* hydrolysate using Flavourzyme® and also found a high level of leucine (111.5 mg/g protein) and the non-essential amino acid arginine (182.0 mg/g protein). The content of hydrophobic amino acids was high, accounting for nearly 43.8% of total amino acids. The abundance of hydrophobic amino acids has been found to affect the antioxidant activity of protein hydrolysates. Moreover, the amino acid profile of the SFS protein extract was found to meet the recommended pattern of amino acid requirements for elderly people, as designated by FAO/WHO (2013).

DPPH radical scavenging assay

Antioxidants play a vital role in protecting skin from UV exposure and aging. Free radical scavengers prevent UV-induced skin damage through their inhibition of the expression and activity of matrix metalloproteinases (MMPs). They also stimulate the inflammatory process, reflect the degree of oxidative stress, and maintain a normal physiologic state [29]. Recently, several studies have been conducted to enhance bioactive peptides present in seafoods and its fermented products [30,31]. In the present study, we found that the DPPH radical scavenging activity of SFSC extract increased with protein concentration (Fig. 1). The radical

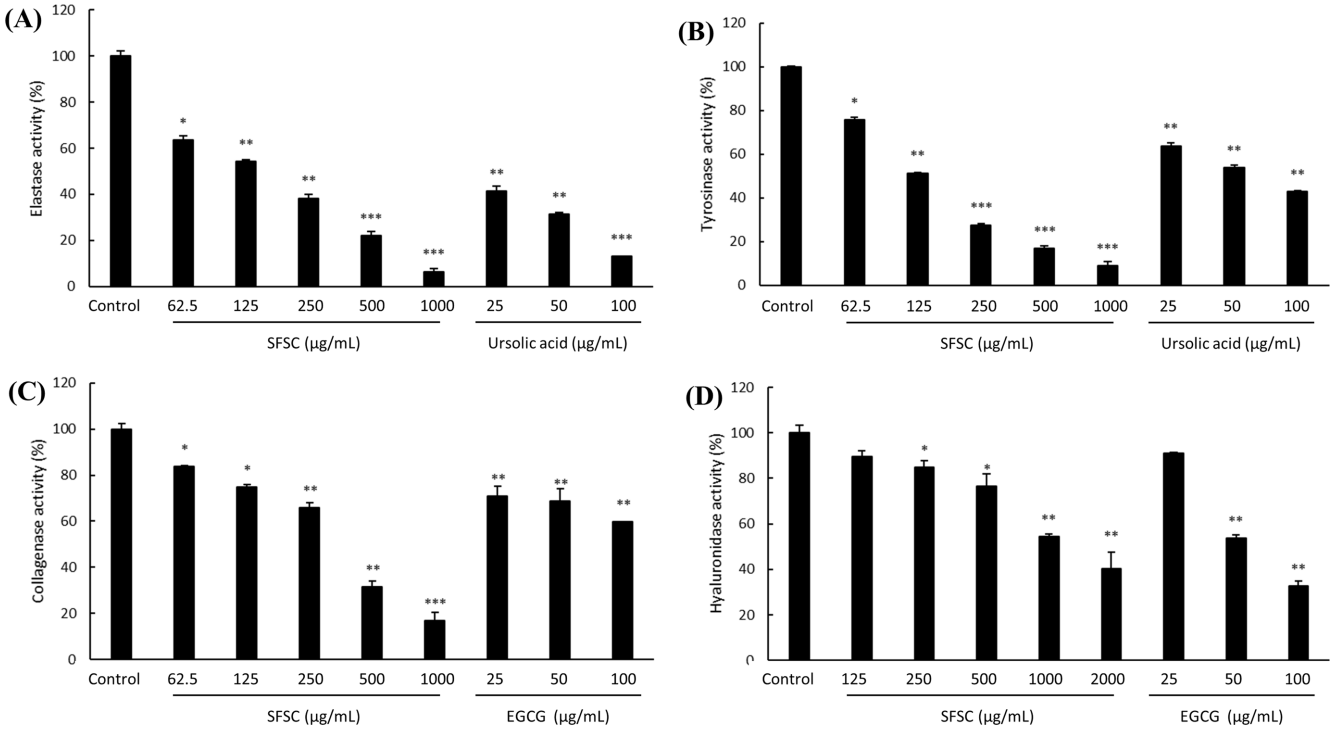

Fig. 2 Comparison of anti-aging skin activity between salted and fermented shrimps and positive control. (A) elastase activity. (B) tyrosinase activity. (C) collagenase activity. (D) hyaluronidase activity. All data are presented as the mean ± SD of triplicate results. *represents p <0.05; **represents p <0.01, ***represents p <0.001 vs control)

scavenging effect at 6.25-100 μg/mL ranged from 15.32 to 56.99%. BHA, a potent antioxidant compound, exhibited weak activity when compared with SFSC protein extract, similar to the value reported by [31]. These results support previous studies on antioxidant activities found in paste and raw materials obtained from shrimp [22]. They reported that DPPH radical scavenging activity of protein hydrolysates from *A. indicus* were 7.04 μM Trolox/g. Furthermore, the activity obtained for SFSC was similar to that reported for silk sericin protein hydrolysates [32]. Scavenging potential is influenced by the amino acid composition. A high content of Lys and Asp has been found in many other antioxidant peptides [33]. This study appears to be the first reporting antioxidant peptides from fermented shrimp paste.

Anti-aging inhibitory activities

The inhibitory activity of SFSC on elastase and tyrosinase was compared to that of ursolic acid, which has been shown to be an effective agent for protecting skin cells against UVB. As shown in the Figure 2A, SFSC inhibited the activity of elastase in a dose-dependent manner. SFSC concentrations of 62.5, 125, 250, 500, 1000 μg/mL inhibited elastase activity by 36.55±2.17, 45.77±1.81, 61.7±0.87, 77.87±1.62, and 93.52±1.91%, respectively. The amount needed for 50% inhibition (IC_{50}) was 182.76±12.38 μg/mL. Ursolic acid, the positive control, demonstrated effective elastase inhibition. The inhibition of tyrosinase by SFSC is shown in Fig. 2B, and was also dose dependent. The highest inhibitory activity of SFSC on tyrosinase was 91.03±8.93% at 500 μg/mL, compared to 57.21% at 100 μg/mL for ursolic acid. The inhibitory activity of SFSC on collagenase ranged from 24.32-91.03% at 62.5-1000 μg/mL, respectively, while epigallocatechin gallate inhibited collagenase by 36.23-57.21% at the concentrations tested. The IC_{50} of hyaluronidase inhibition for the SFSC sample was 1447.95 ±28.92 μg/mL.

Several studies have reported that fermentation increases the anti-aging activity of products [34,35]. Jang, Kim et al. [34] reported greater values for fermented *Trapa japonica* fruit than for protein extracted from fresh fruit. The results correspond to earlier findings of Fan, Zhuang et al. [36] and Chlapanidas, Faragò et al. [37] for protein extracts of marine organisms, both *in vitro* and *in vivo*. A study conducted by Abdillah, Wijayanti et al. [38] found that hydrolyzed collagen from sea cucumbers had an IC_{50} value of 125 μg/mL.

Anti-aging activity may be due to the presence of various amino acids in protein extracts. Detailed knowledge of the molecular mechanisms underlying the effect of peptides on the aging of skin is limited. However, the increase in anti-aging activity found in protein hydrolysates might be explained by the fact that fermentation of protein is shown to be necessary for the release of active peptides from inactive protein, or the synthesis of new peptides by microorganisms [30]. The anti-photoaging effect of hexapeptide-11, a peptide purified from *Saccharomyces* yeast fermentation, was evaluated both *in vitro* and in a clinical trial of 25 healthy

Table 3 Sensory evaluation of salted and fermented shrimp products*

Free amino acid	SFS	SFSC
Softness	4.56±0.15	4.61±0.32
Fibrousness	3.12±0.11	3.25±0.13
Sweetness	1.66±0.52	1.67±0.41
Bitterness	1.30±0.25[a]	2.20±0.21[a]
Sourness	2.56±0.16	2.25±0.11
Aromaintensity	7.85±0.13[a]	7.15±0.15[b]
Aftertaste	6.56±0.07	6.54±0.24
Accept ability	9.41±0.27	9.37±0.18

*Values presented are means of triplicates ± SD
[a,b]The different letters in the same line represent that there are significant differences at the level of $p < 0.05$

volunteers [39]. However, further study is required in the identification of bioactive peptides. Analysis of the amino acid composition of peptides responsible for the anti-wrinkle effect would provide insight into the evaluation or synthesis of new peptides that could ultimately replace conventional proteins in skin cosmetics.

Sensory evaluation

The results of sensory evaluation showed no difference in overall acceptability between conventional (SFS) and *S. cibaria*-fermented (SFSC) salted shrimps (Table 3). In comparison to SFS, SFSC scored higher for softness, fibrousness, and bitterness. Overall, *S. cibaria* fermentation did not change the sensorial characteristics of salted shrimp. Moreover, based on the results of the antioxidant and anti-wrinkle tests, this study demonstrates that salted shrimp subjected to fermentation by *Salinivibrio cibaria* is an important source of bioactive peptides that can potentially be used to develop nutricosmetic products.

In conclusion, this study suggests that the use of *Salinivibrio cibaria* BAO-01 as a starter culture in salted fermented shrimp may be a good strategy to enhance antioxidant and anti-wrinkle activities during fermentation. Concerning the sensory profile, the salted fermented shrimp s has an apparent positive impact. The outcomes of our study would be of practical interest to fermented shrimps, especially when the option for developing functional products through the selection of microorganism may be an option to produce fermented shrimp with potential new profiles. However, further researches on the safety properties and especially the impact of with long-term use of different shrimp varieties and concentration will be necessary.

Acknowledgment This study was carried out with the support of the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2017R1D1A3B 03027816).

Conflict of interest statement We declare that we have no conflicts of interest.

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