Food Chemistry xxx (xxxx) xxxx

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidant mechanism, antibacterial activity, and functional characterization of peptide fractions obtained from barred mackerel gelatin with a focus on application in carbonated beverages

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ARTICLE INFO

Keywords:
Antioxidant mechanism
Antibacterial peptides
Carbonated beverages
Gelatin peptides

ABSTRACT

The present study aimed to use fish by-products to generate gelatin peptides with potential applications in carbonated beverages. After ultrafiltration, the F < 3 kDa (fraction < 3 kDa) showed the highest peptide concentration (227.22 mg/g) as well as antibacterial (MIC of ≤ 0.5 mg/mL) and antioxidant activities, including hydroxyl and superoxide radical scavenging, ferrous chelation, and ferric reduction (with IC_{50} values of 0.88, 1.04, 0.50 mg/mL, and 0.58, respectively). 2,2-diphenyl-1-picrylhydrazyl scavenging was the highest in the 3 < F < 10 kDa (IC_{50} of 0.64 mg/mL). *In vitro* gastrointestinal digestion significantly decreased all biological activities. Solubility, water holding capacity, and emulsifying activity of the F < 3 kDa were the highest while foaming properties and overfoaming were reversibly related to the molecular weight. All abovementioned properties, in addition to *in vitro* cytotoxicity analysis in different cell lines and better flavor characteristics, indicated that the F < 3 kDa could be safely and properly used as an ingredient for the fortification of carbonated beverages.

1. Introduction

Since fish gelatin is obtained as a by-product, the value added from bioactive peptides generated from enzymatic hydrolysis of fish gelatin could be a promising method to fortify food products such as carbonated beverages. The demand for beverages enriched with bioactive compounds, especially proteinaceous ones, due to the requirements of the human body for essential amino acids (EAAs), is encouraging researchers and manufacturers to identify new sources of proteins that are highly digestible, biologically active, and rich in EAAs. The use of fish proteins can meet an important demand for protein sources with health effects (Egerton et al., 2017). In some cases beverages have been fortified by proteinaceous components. In a research performed by Egerton, Culloty, Whooley, Stanton, & Ross, 2018, fish protein was hydrolyzed by different commercial proteases that revealed a good source for beverage fortification. In a research conducted Yadav, Vishwakarma, Borad, Bansal, Jaiswal & Sharma (2016) mango ready-to-serve beverage was enriched by modified whey protein. The authors stated that the mango beverage with 3.0% hydrolysed whey protein showed good sensory and stability as well storage properties.

Finding novel and safe proteases approved by global organizations and related to healthcare protocols is an important part of the protein and peptide science called “proteomics” and “peptidomics.” Actinidin is a member of the papain-like family of cysteine proteases (Grozdanovic et al., 2016), which can be used alone or in combination with other proteases to generate bioactive peptides from different protein sources. In the present study, gelatin was hydrolyzed by the sequential application of Alcalase® (a microbial protease obtained from *Bacillus licheniformis*) and actinidin (obtained from kiwifruit). Previous studies have focused on using bioactive peptides to produce functional beverages (Karnjanapratum & Benjakul, 2017), however, using gelatin derived peptides with especial pharmaceutical characteristics has been neglected. Albeit, there is a research field that investigates the encapsulation of these peptides using biopolymers as surveyed by Ramezanzade, Hosseini, & Nikkhah, (2017). This study revealed that rainbow trout (*Oncorhynchus mykiss*) skin-derived antioxidant peptides were encapsulated in chitosan-coated liposomes. Investigation of encapsulation procedure could be a promising approach for food and

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https://doi.org/10.1016/j.foodchem.2020.128339
Received 17 August 2020; Received in revised form 16 September 2020; Accepted 6 October 2020

beverage fortifications especially in order to cover the bitter taste of peptides.

According to food and agriculture organization of united nation reports (FAO), the global capture production of barred mackerel (*Scomberomorus commerson*) was estimated to be 275.606 tons in 2016, which is a huge amount considering that > 50% is discarded as by-products (Mirzapour-Kouhdasht, Moosavi-Nasab, Krishnaswamy, & Khalesi, 2020). To the best of our knowledge, no previous study has reported the use of these by-products and their commercialization.

The purpose of this study was to generate bioactive peptides from barred mackerel by-product (skin, scales, and fins) gelatin using a novel enzymatic strategy and investigate the antioxidant mechanism, antibacterial activity, and potential application of the peptides in carbonated beverages.

2. Materials and methods

Barred mackerel (length of 30–85 cm and weight of 4–8 kg) and plain sparkling water (Seagram's Sparkling water) were procured from the local market in Gwangju, South Korea. Alcalase® (406.80 U·mg^{-1}) was purchased from the National Institute of Genetic Engineering and Biotechnology, Tehran, Iran. Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI)-1640 medium were purchased from Corning (Mediatech Inc., Manassas, VA, USA). The cell lines Caco-2 (human colon epithelial), RAW264.7 (murine macrophage), and L929 (mouse fibroblast, subcutaneous connective tissue) were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK). All chemicals and reagents were of analytical grade and obtained from Sigma Aldrich, Inc. (St. Louis, MO, USA).

2.1. Protein extraction and hydrolysis

Gelatin production and hydrolysis protocols were performed according to the optimum conditions obtained previously (Mirzapour-Kouhdasht, Moosavi-Nasab, Krishnaswamy, & Khalesi, 2020). Briefly, by-products (skin, scales, and fins) were cut into small pieces (2 cm^2) and swollen in NaOH solution (pH 13) for 3 h. After neutralization, the swollen by-products were heated in hot water (70.71 °C) for 5.85 h. Subsequently, the solution was filtered through two-layers of cheesecloth and the permeate was lyophilized as gelatin powder.

For the hydrolysis, a 2.5% (w/v) gelatin solution was prepared in Tris-HCl, pH 8.5 and 7.5 for Alcalase® (406.80 U·mg^{-1}) and actinidin (174.26 U·mg^{-1}), respectively. The hydrolysis reaction was initiated with Alcalase® at optimum conditions (stirred at 80 rpm and 55 °C for 3 h), followed by actinidin (stirred at 80 rpm and 37 °C for 3 h). The pH was checked every 1 min for the first 1 h of the enzymatic reactions and every 5 min after that. The pH was adjusted using 0.1 N NaOH or 0.1 N HCl. Eventually, the peptides were separated by centrifugation at 8000 × *g* for 15 min at 10 °C, after deactivation of the enzymatic reaction by boiling for 20 min.

2.2. Ultrafiltration

The bioactive peptides were passed through ultrafiltration units with a molecular weight cutoff (MWCO) of 3, 10, and 30 kDa. All fractions were investigated for their antioxidant activity using different assays. The most active fraction was then lyophilized and kept at −20 °C for further purification. Fractions tested were termed F < 3, 3 < F < 10, and 10 < F < 30, depending on the size of the peptides in the fraction.

2.3. Peptide concentration

The concentration of peptides was determined using a spectrophotometric method (Wang et al., 2008). Samples (50 μL) were added to 2 mL of o-phthaldialdehyde (OPA) mixture (containing 1 mL 100 mM sodium tetra borate, 0.1 mL 20% (w/w) sodium dodecyl sulfate (SDS), 1.6 mg OPA dissolved in 40 μL methanol, 4 μL of β-mercaptoethanol, and 850 μL distilled water). The absorbance of samples was recorded at 340 nm, after 4 min at ambient temperature. Glutathione was used to plot a standard curve to measure peptide concentration. The degree of sample hydrolysis was calculated by dividing the sample peptide concentration by the sample protein concentration, which was obtained by a micro Kjeldahl apparatus (PECOFOOD, PDU-500S, Iran), and multiplied by 100. Experiments were performed in triplicate

2.4. Amino acid analysis

The amino acid analysis of gelatin and different fractions obtained from the ultrafiltration of its hydrolysates was performed as described by Siswoyo, Mardiana, Lee, & Hoshokawa, (2011), with modifications described by Mirzapour-Kouhdasht, Moosavi-Nasab, Krishnaswamy, & Khalesi (2020). A 50 mL aliquot was hydrolyzed in a vacuum-sealed glass tube with 6 M HCl containing 0.1% phenol (at 110 °C, for 24 h). Norleucine (Sigma Aldrich, Inc., St. Louis, MO, USA) was used as the internal standard. Subsequently, the amino acid composition was analyzed using an amino acid analyzer apparatus (HITACHI 8900 Amino Acid Analyzer, Japan).

2.5. Antibacterial analysis

The antibacterial activity of different fractions against *Escherichia coli* ATCC 8739 was determined using the agar dilution method, with some modifications. Summarily, 50 μL of different concentrations of fractions (0.5–10 mg/mL) were aliquoted into a 96-well microplate containing Mueller Hinton broth medium. Next, an aliquot of the resazurin indicator (0.02%) was added to each well. An equal volume of bacterial suspension with a cell density of 0.5 McFarland and Mueller Hinton broth were inoculated in each well. The microplate was incubated at 37 °C for 24 h. Sterile medium and gentamycin (20 μg/mL) were used as negative and positive controls, respectively. The minimum inhibitory concentration (MIC) was estimated as the lowest concentration of peptides that could inhibit bacterial growth.

2.6. Antioxidant mechanism

2.6.1. 2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging

The DPPH radical scavenging of the fractions was determined according to the method of Ambigaipalan and Shahidi (2015). An aliquot (200 μL) of peptide fractions was added to 0.1 mM DPPH in 95% methanol (with a sample to DPPH solution ratio of 1:4 v/v). After 30 min of incubation in the dark, the absorbance was recorded at 517 nm using a microplate reader (BioTek Instruments, Winooski, VT, USA). Methanol (95%) was the control, and the procedure was the same as mentioned above. Distilled water and ascorbic acid were used as negative and positive controls, respectively, to confirm the method. DPPH radical scavenging was calculated using equation (1).

$$\text{DPPH radical scavenging}(\%) = (\text{Ac}-\text{As}/\text{Ac}) \times 100 \quad (1)$$

where Ac and As are the absorbance of the control and sample, respectively.

2.6.2. Ferrous chelation

The ferrous chelating capacity of gelatin hydrolysates was determined as described by Ambigaipalan, Al-Khalifa, and Shahidi (2015). Briefly, the gelatin hydrolysates (200 μL) were dissolved in distilled water (1.74 mL), followed by adding 2 mM $FeCl_2$ (20 μL) and 5 mM ferrozine (40 μL) to the mixture. After thoroughly shaking the mixture, the samples were kept at room temperature for 10 min, following which the absorbance of the mixture was recorded at 562 nm. A standard curve was plotted using Na_3EDTA to determine the metal chelating activity using equation (2). The IC_{50} was calculated as the

concentration of peptides that could chelate 50% of ferrous ions.

$$\text{Ferrous chelating activity}(\%) = [1 - (\text{As}/\text{Ac})] \times 100 \quad (2)$$

where As and Ac are the absorbance of the sample and control, respectively.

2.6.3. Ferric reduction

The reducing capacity of peptides was determined using a spectrophotometric method (Yen & Chen, 1995). Different concentrations of hydrolysates were added to a 1% potassium ferricyanide solution in the ratio of 1:1 (v/v). After incubation at 50 °C for 20 min, 10% trichloroacetic acid (2.5 mL) was added followed by centrifugation at 3000 × *g* for 10 min. Next, an aliquot of the supernatant of each sample was added to distilled water (1:1 v/v) and 0.5 mL ferric chloride (1%) was added to the mixtures. The mixtures were kept at room temperature for 10 min before the determination of the absorbance at 700 nm. A standard curve was prepared using 0–500 μM of Trolox.

2.6.4. Hydroxyl radical scavenging

An aliquot of 1,10-phenanthroline solution (1.865 mM) was mixed in the ratio of 1:2 with the sample (2.0 mL). A $FeSO_4{\cdot}7H_2O$ solution (with the same volume and concentration of the 1,10-phenanthroline) was then added to the mixture. Next, H_2O_2 (1.0 mL, 0.03% v/v) was added to the mixture to initiate the reaction. The mixture was then incubated at 37 °C for 1 h followed by determination of the absorbance at 536 nm. The reaction mixture without antioxidant and H_2O_2 were used as the negative control and blank, respectively. The hydroxyl radical scavenging percentage was calculated using Eq. (3). The IC_{50} was calculated as the concentration of the peptides that could scavenge 50% of the hydroxyl radicals.

$$\text{Hydroxyl radical scavenging}(\%) = [(\text{As} - \text{An})/(\text{Ab} - \text{An})] \times 100\% \quad (3)$$

where As, An, and Ab are the absorbance of the sample, negative control, and blank, respectively (Wang et al., 2013).

2.6.5. Superoxide radical scavenging

The superoxide radical scavenging capacity was measured as described by Xie et al. (2016), with minor modifications. Samples (1 mL) were added to 10 mL Tris-HCl buffer (50 mM, pH 8.2) and stirred at 25 °C for 20 min. Subsequently, 1 mL pyrogallol (3 mM) was added and the absorbance (325 nm) of the mixtures was precisely recorded for 5 min to determine the change in absorbance (A/min) every 10 s. Distilled water was used as the blank. The superoxide radical scavenging activity was calculated using equation (4). The IC_{50} was calculated as the concentration of the peptides that could scavenge 50% of the superoxide radicals.

$$\text{Superoxide radical scavenging activity}(\%) = (1 - A_1/A_0) \times 100 \quad (4)$$

where A_1 and A_0 are the absorbance change of the samples and blank, respectively.

2.7. In vitro gastrointestinal digestion

An *in vitro* gastrointestinal digestion assay was conducted as described by Lajmi, Gómez-Estaca, Hammami, & Martínez-Alvarez (2019), with modifications. Briefly, a solution of each fraction (10 mg/mL) was prepared in distilled water with a pH of 2 (adjusted with 3 N HCl). Subsequently, pepsin was added to the mixture with an enzyme to substrate ratio of 1:2, followed by incubation at 37 °C for 1 h (with shaking at 100 rpm). Next, the pH was increased to 5.3 using a saturated solution of $NaHCO_3$ and a final pH of 7.5 was achieved using 3 N NaOH. Thereafter, pancreatin was added to the mixture with the same enzyme to substrate ratio, followed by incubation for 2 h at the same temperature and shaking speed. The intestinal digestion was terminated by heating for 2 min at 95 °C. The effects of gastrointestinal digestion on the bioactivity of fractions were evaluated using antioxidant and antibacterial assays.

2.8. Cytotoxicity

A cytotoxicity test was performed using the method described by Benjakul, Karnjanapratum, & Visessanguan (2018), with some modifications. Cubic bottles (25 cm^3) were used to culture the Caco-2 and L929 cell lines in DMEM. To culture the RAW264.7 cell line, RPMI medium was used. All media were supplemented with 10% fetal bovine serum. The cultured cell lines were then incubated at 5% CO_2 and a temperature of 37 °C.

Next, 100 μL of each cell line culture, with a cell density of 2×10^4 cells/mL, was seeded onto microplate wells. An equal volume of each fraction with different concentrations (2, 4, and 6 mg/mL) was then added to the wells. The microplate was incubated for 72 h in the same conditions, followed by a cell viability assay using the MTT I proliferation kit (Roche Diagnostics; Burgess Hill, West Sussex, UK). The absorbance was determined at 570 nm using a microplate reader.

2.9. Functional properties for carbonated beverage fortification

2.9.1. Solubility

The solubility of gelatin and the peptides was determined under different pH values ranging from 3 to 9 (Zhao, Xiong, & McNear, 2013). The sample solutions with a concentration of 10 mg/mL were prepared using 25 mM phosphate buffers followed by centrifugation at 5000 × *g* for 10 min. The solubility of each sample was calculated using equation (5).

$$\text{Solubility}(\%) = (\text{Protein content of supernatant}/\text{Protein content of sample}) \times 100 \quad (5)$$

The protein content of the supernatant and samples was determined using the micro Kjeldahl method.

2.9.2. Water holding and fat-binding capacities

The water-holding capacity (WHC) and fat-binding capacity (FBC) were determined as described by Zayas (1997), with slight modifications. Sample (100 mg) was weighed in a tube and diluted with 10 mL distilled water. Subsequently, the tube containing the sample was kept at room temperature for 1 h, with 10 min shaking intervals. Next, the sample was centrifuged at 2000 × *g* for 25 min and drained on a filter paper. The WHC was calculated using the ratio between the tube weight before and after draining.

To determine the FBC, sample (100 mg) was weighed in a tube and mixed with 2 mL olive oil. The mixture was kept for 30 min at room temperature, with 10 min shaking intervals. Next, the sample was centrifuged at 2000 × *g* for 25 min and drained on a filter paper. The FBC was calculated using the ratio between the tube weight before and after draining.

2.9.3. Emulsion activity and stability

The emulsion activity index (EAI) and emulsion stability index (ESI) are two important factors affecting the potential application of the peptides in carbonated drinks. To determine the EAI and ESI, a volume (30 mL) of each fraction (0.1% w/v) was mixed with 10 mL soybean oil using a dispenser/homogenizer (HG-15D, Daihan Scientific, Seoul, Korea). After 0 and 10 min of homogenizing, 100 μL of the emulsion was diluted 100-fold using an SDS solution (0.1% w/v). The absorbance of the samples was recorded at 500 nm using a microplate reader. The EAI and ESI factors were determined using Eqs. (6) and (7).

$$\text{EAI}\ (m^2/g) = (2 \times 2.303 \times A)/(0.25 \times Cp) \quad (6)$$

$$\text{ESI}(\text{min}) = A_0 \times \Delta t/\Delta A \quad (7)$$

where A, Cp, A_0, and A_{10} are the absorbance at 500 nm, protein

concentration, and absorbance at time 0 and 10 min after homogenization, respectively. Δt is 10 min and ΔA is the difference between A_0 and A_{10}.

2.9.4. Foam expansion and stability

Foam expansion (FE) and stability (FS) are two important factors, especially in food systems containing proteins and peptides. After homogenizing sample solutions (2% w/v) for 1 min at room temperature using an Ultra-turax (IKAT-25, Cole Parmer, USA), samples were translocated into a cylinder (Shahidi, Han, & Synowiecki, 1995). The change in sample volume was determined at 30 and 60 min. The FE and FS values were determined using Eqs. (8) and (9), respectively.

$$FE(\%) = \frac{Totalvolumeafterhomogenizing - Volumebeforehomogenizing}{Volumebeforehomogenizing} \times 100 \quad (8)$$

$$FS(\%) = \frac{Totalvolumeafter30and60min - Volumebeforehomogenizing}{Volumebeforehomogenizing} \times 100 \quad (9)$$

2.9.5. Gushing test

Overfoaming was calculated by the procedure described by Deckers et al. (2012), with modifications. Briefly, plain sparkling water bottles (350 mL) were divided into four groups and weighed. In each group, 20 mL of sparkling water was replaced by an equal volume of peptides (2 mg/mL in phosphate buffer, pH 6) in bottles and one bottle was used as the control (phosphate buffer instead of peptide). Next, the bottles were left standing for 10 min after shaking horizontally for 3 days at 150 rpm. Subsequently, the bottles were laid down once to the left side and once to the right side and were left standing for another 10 min. This step was repeated two more times, and after the last one, the bottles were left standing for 30 s and opened. Finally, the bottles were weighed again and the overfoaming was calculated from the differences in weights.

2.9.6. Flavor evaluation

Flavor evaluation was examined using five trained panelists who were first presented with distilled water. The panelists then scored the sample containing different concentrations of hydrolysates (1, 1.5, and 2 mg/mL) in distilled water, as follows:

Score 1: no fishy flavor, Score 2: threshold fishy flavor, Score 3: slight fishy flavor, Score 4: moderate fishy flavor, Score 5: strong fishy flavor, and Score 6: extreme fishy flavor (Stelzleni & Johnson, 2008).

2.9.7. Color

The color of gelatin and hydrolysate samples were analyzed as described in a previous study (Kouhdasht, Moosavi-Nasab, & Aminlari, 2018). The a*, b*, and L* parameters were determined to illustrate the color as a quantitative characteristic.

2.10. Biostatistics

The mean comparison between trials of antioxidant and functional tests was performed using one-way analysis of variance (ANOVA) with IBM SPSS Statistics 25. A confidence level of 95% was applied for the Tukey test.

3. Results and discussion

3.1. Peptide concentration

Peptide concentration is a prominent factor in the determination of the efficiency of hydrolysis, as well as the biological and functional properties of the hydrolyzed products. As shown in Fig. 1-a, the peptide concentration was significantly increased during the first 3 h of hydrolysis and continued to increase until 5 h of hydrolysis. Eventually, the final peptide concentration at 6 h of hydrolysis reached 142 mg/g, which was not significantly ($P < 0.05$) different from that of the concentration at 5 h.

The F < 3 kDa fraction obtained from ultrafiltration showed the highest peptide concentration (227.22 mg/g) among the other fractions (Fig. 2). These results are due to the presence of more amino groups in the lower molecular weight fraction after hydrolysis that can react with OPA. The higher peptide content in the F < 3 kDa fraction compared to the other fractions was likely the reason for the higher biological activities. Liu, Chen, Shao, Wang, & Zhan (2017) suggested that the peptide content affects antioxidant activity. More studies are required to achieve a reasonable relationship between molecular weight and peptide content.

3.2. Amino acid analysis

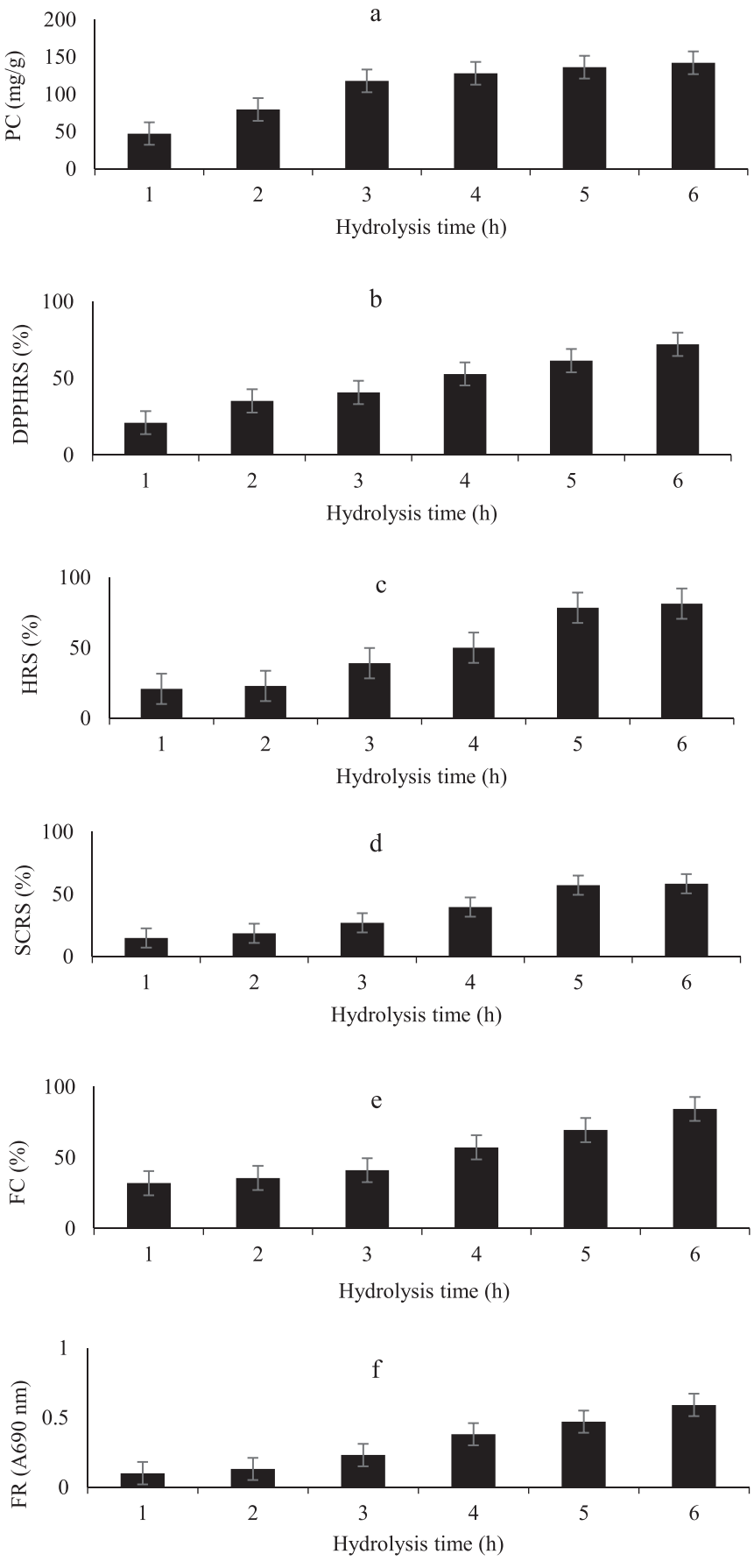
The amino acid analysis of gelatin and different fractions of its hydrolysates obtained from ultrafiltration are shown in Table 1. The major amino acid residues in gelatin and all fractions of its hydrolysates were glycine, proline, and alanine. A research performed by Ramezanzade, Hosseini, & Nikkhah (2017) showed similar results that indicated gelatin hydrolysates rich in glycine, proline, alanine, Glutamic acid and Hydroxyproline. In all peptide fractions, the number of basic amino acids with a positive charge (histidine, arginine, and lysine) was lower than the number of acidic residues with a negative charge (aspartic and glutamic acids). These results showed that the ultrafiltration process with an MWCO of 10 kDa separated some peptides, including small amounts of histidine. The net negative charge of the fraction with the molecular weight < 3 kDa was higher than that of other fractions, which might be the reason for increased antibacterial and antioxidant activities (except for DPPH radical scavenging) of this fraction. Investigations of the structure of anionic peptides with antibacterial activity have indicated that these peptides generally range in net charge from −1 to −7 (with 5–70 residues); some of these peptides require post-translational modifications to show antibacterial activity (Harris, Dennison, & Phoenix, 2009).

An abundance of hydrophobic amino acids (glycine, proline, alanine, valine, leucine, and isoleucine) is a key factor for potent antioxidant activity (Hajfathalian, Ghelichi, García-Moreno, Moltke Sørensen, & Jacobsen, 2018). The increased amount of hydrophobic amino acids in the F < 3 kDa fraction compared to that in other fractions may be the reason for the higher antioxidant activity of this fraction.

3.3. Antioxidant activity

3.3.1. DPPH radical scavenging

The 3 < F < 10 kDa fraction (IC_{50} of 0.64 mg/mL) had significantly higher DPPH radical scavenging ability than the F < 3 kDa (IC_{50} of 2.66 mg/mL) and 10–30 kDa fractions (IC_{50} of 2.39 mg/mL). The decrease in DPPH radical scavenging activity of the hydrolysates after fractionation using 3 kDa filters might be due to the removal of bioactive peptides with the special activity of scavenging these radicals. As our parallel study (Mirzapour-Kouhdasht, Moosavi-Nasab, Krishnaswamy, & Khalesi, 2020) demonstrated, a bioactive peptide with a molecular weight of 3,023.38 g/mol was identified from the 3 < F < 10 kDa fraction. In a study performed by Yang, Ho, Chu, & Chow (2008), *Rachycentron canadum* skin gelatin was hydrolyzed. The results of this study indicated that ultrafiltration by molecular weight cut-offs of 10, 5, and 3 kDa significantly enhanced DPPH radical scavenging activity. Indeed, different types of proteolytic enzymes result in peptides with different bioactivity. In this regard, in a previous study, the pigeon pea protein was hydrolyzed using food grade enzymes, including Alcalase®, pancreatin, and pepsin-pancreatin (Olagunju, Omoba, Enujiugha, Alashi, & Aluko, 2018); the analysis of all three hydrolysates after filtration through 1, 3, 5, and 10 kDa ultrafilters indicated that the fractions with higher molecular weight (> 10 kDa) had stronger DPPH radical scavenging activity. A possible reason for such outcomes could be the removal of peptides with higher molecular weights that have strong DPPH radical activity. It also may



(caption on next page)

Fig. 1. The Peptide concentration (a), DPPH radical scavenging (b), hydroxyl radical scavenging (c), superoxide radical scavenging (d), ferrous chelating (e), and ferric reducing (f) during 6 h of hydrolysis.

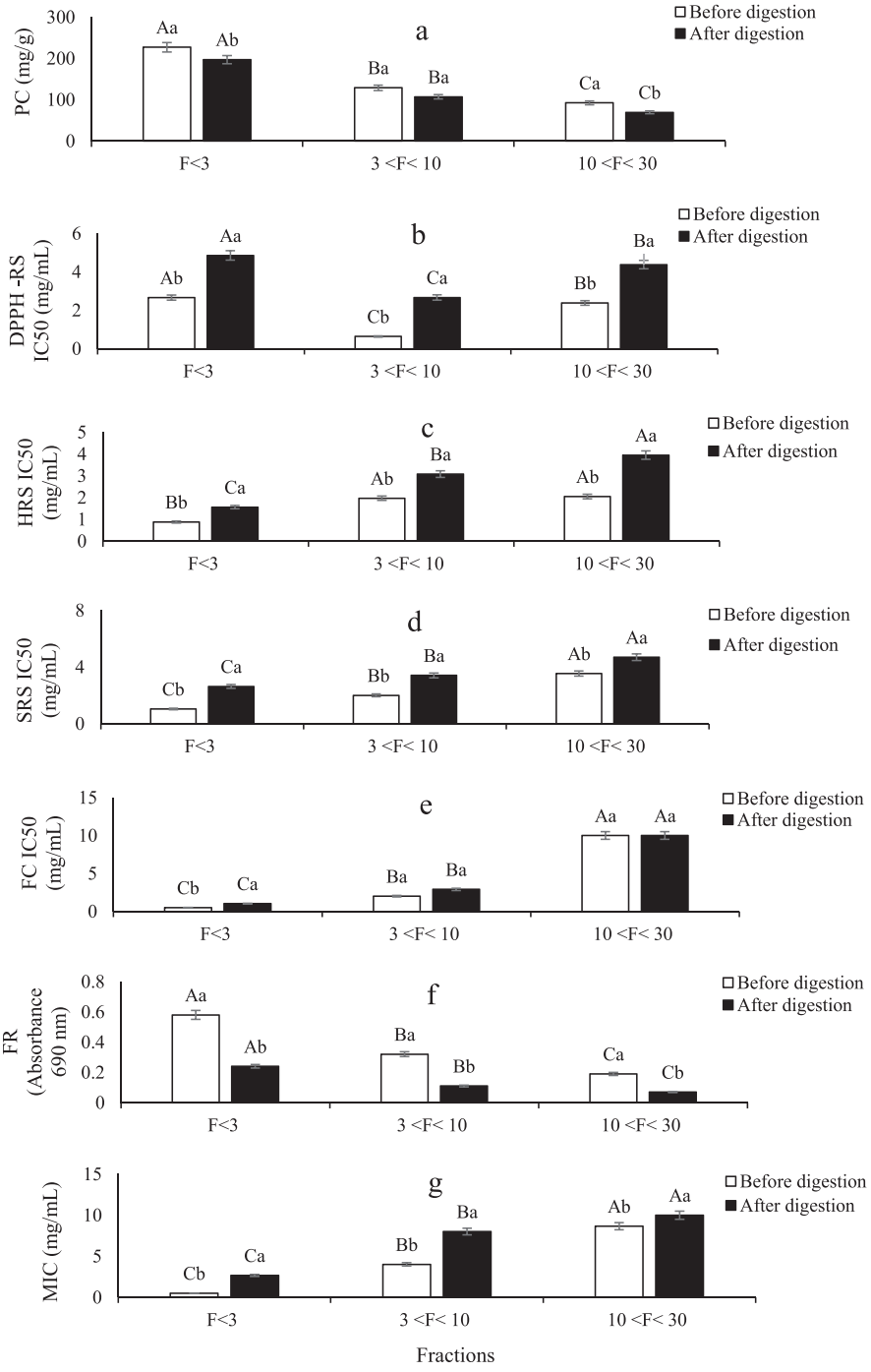


Fig. 2. The effect of *in vitro* gastrointestinal digestion on bioactivity of fractions. Peptide concentration (a), DPPH radical scavenging (b), hydroxyl radical scavenging (c), superoxide radical scavenging (d), ferrous chelating (e), ferric reducing (f), and minimum inhibitory activity against *E.coli* (g). Different uppercase and lower case letters represent the significant differences in different fractions and different treatments, respectively ($P < 0.05$).

be because of hydrophobicity of peptides, because DPPH is a hydrophobic radical that works well in systems with hydrophobic state (Li, Jiang, Zhang, Mu, & Liu, 2008).

3.3.2. Hydroxyl radical scavenging

Hydroxyl radicals are one of the most destructive reactive oxygen species compounds due to their reaction with biological macromolecules such as DNA and proteins. One of the most important factors affecting antioxidant activity is the hydrophobic amino acid content, especially glycine, proline, and alanine (Rajapakse, Mendis, Jung, Je, & Kim, 2005). As previously mentioned, the major amino acids in all fractions were glycine, proline, and alanine. Especially, the higher amount of proline in the F < 3 kDa fraction (16.26%) compared to that in other fractions (14.08% and 12.16% for the

Table 1
Amino acid composition of different ultrafiltration fractions of gelatin peptides (number of amino acid in 100 residues).

Amino acid	UF fractions			
	Gelatin	F < 3 kDa	3 < F < 10	10 < F < 2.4632.30
Asp	4.20 ± 0.10	5.18 ± 0.04	4.46 ± 0.22	4.83 ± 0.00
Glu	5.25 ± 0.32	9.46 ± 0.94	8.17 ± 0.09	8.05 ± 0.12
Ser	2.90 ± 0.06	2.67 ± 0.01	2.98 ± 0.01	3.00 ± 0.03
His	1.72 ± 0.01	–	–	1.08 ± 0.10
Gly	34.40 ± 0.30	32.21 ± 0.05	32.18 ± 0.10	26.16 ± 0.14
Thr	2.60 ± 0.06	2.83 ± 0.08	1.75 ± 0.02	3.67 ± 0.02
Arg	4.70 ± 0.05	6.00 ± 0.00	5.46 ± 0.00	5.20 ± 0.01
Ala	11.70 ± 0.02	11.81 ± 0.05	11.27 ± 0.03	9.63 ± 0.05
Tyr	0.60 ± 0.10	0.55 ± 0.02	1.00 ± 0.02	1.50 ± 0.35
Cys	–	–	–	–
Val	1.50 ± 0.05	1.47 ± 0.01	1.02 ± 0.01	0.43 ± 0.03
Met	1.00 ± 0.02	0.98 ± 0.03	1.25 ± 0.14	1.36 ± 0.07
Phe	2.80 ± 0.01	2.92 ± 0.04	2.44 ± 0.08	2.67 ± 0.19
Ile	4.50 ± 0.04	4.57 ± 0.01	4.33 ± 0.05	4.28 ± 0.02
Leu	2.40 ± 0.02	2.52 ± 0.03	2.07 ± 0.03	1.41 ± 0.00
Lys	3.70 ± 0.01	2.02 ± 0.12	3.00 ± 0.02	2.85 ± 0.04
Pro	15.65 ± 0.07	16.26 ± 0.16	14.08 ± 0.11	12.16 ± 0.05

Data are shown as mean ± SD of three replicates. Different superscript letters in the same column represent the significant difference ($P < 0.05$).

3 < F < 10 and 10 < F < 30 kDa fractions, respectively) could be the reason for the higher hydroxyl radical scavenging activity of this fraction. These results revealed that the F < 3 kDa fraction, with an IC_{50} of 0.88 mg/mL, had significantly higher hydroxyl radical scavenging activity than the other fractions (as shown in Fig. 2-c) (with IC_{50} values of 1.97 and 2.05 mg/mL for the 3 < F < 10 and 10 < F < 30 fractions, respectively). Moreover, no significant ($P > 0.05$) difference was observed in hydroxyl radical scavenging activity between the 3 < F < 10 and 10 < F < 30 fractions. There are many studies whose findings are consistent with these results. Sun, Shen, and Luo (2011) investigated the antioxidant activity of different fractions of the porcine hemoglobin hydrolysates. Their results indicated that after filtering hydrolysates through 10, 5, and 3 kDa filters, the hydroxyl radical scavenging activity of the F < 3 kDa fraction was significantly higher than that of the others.

3.3.3. Superoxide radical scavenging

The formation of free radicals is an unpleasant result in aerobic systems; superoxide anion ($\cdot O^{2-}$) is one of the dangerous metabolic by-products of aerobic organisms (Wang, Liao, Thakur, Zhang, Huang, & Wei, 2019). The results of the superoxide radical scavenging activity of different fractions (Fig. 2) revealed that the F < 3 kDa fraction (with an IC_{50} of 1.04 mg/mL) had significantly ($P < 0.05$) higher activity than the 3 < F < 10 kDa (IC_{50} of 2.00 mg/mL) and 10 < F < 30 kDa (IC_{50} of 3.54 mg/mL) fractions. Furthermore, the difference between the 3 < F < 10 and 10 < F < 30 kDa fractions was statistically significant ($P < 0.05$). These results could be due to the higher arginine content in the F < 3 kDa (6.00%) and 3–10 kDa (5.46%) fractions compared to the 10 < F < 30 kDa fraction (5.20%). Arginine is one of the key factors affecting the protective strength of antioxidant agents against reactive oxygen species such as superoxide anion radicals (Lass, Suessenbacher, Wölkart, Mayer, & Brunner, 2002).

3.3.4. Ferrous chelation

Ferrous chelating analysis is a widely used procedure to evaluate the antioxidant potential of protein hydrolysates and is affected by the molecular weight of the peptide fractions. The results of the ferrous chelating activity in the present study (Fig. 2) demonstrated that this activity of the F < 3 kDa fraction (with an IC_{50} of 0.50 mg/mL) was significantly ($P < 0.05$) higher than that of the 3 < F < 10 kDa and 10 < F < 30 kDa fractions (IC_{50} values of 2.00 and 10.00 mg/mL, respectively).

Sabeena Farvin et al. (2014) reported that the < 3 kDa fraction obtained from Cod (*Gadus morhua*) protein hydrolysates had significantly higher ferrous chelating activity than the 3–5 kDa and > 5 kDa fractions (at concentrations < 1 mg/mL).

Feng and Mine (2006) investigated hen egg yolk phosvitin hydrolysates. The results of this study indicated that phosphopeptides with a molecular weight of 1–3 kDa exhibited higher metal chelating activities than those of the unfractionated phosvitin hydrolysates. To the best of our knowledge there is no certain relationship between molecular weight and ferrous chelating activity. However, all studies have mentioned that the metal chelating activity of peptides is highly related to the amino acid sequence and composition of peptides. Another important factor is the increase in concentration of metal chelating peptides during passing through ultrafiltration units.

3.3.5. Ferric reduction

The ferric reducing activity of a peptide is positively correlated with its antioxidant activity. The results of the ferric reducing power of the different fractions (Fig. 2) indicated similar results as the hydroxyl radical scavenging, superoxide radical scavenging, and ferrous chelating activity. The highest ferric reducing power (0.58) was found in the F < 3 kDa fraction. Moreover, the reducing activity of the 3 < F < 10 fraction (0.32) was significantly higher than that of the 10 < F < 30 kDa fraction (0.19). The reason for these results may be due to the higher leucine content in the F < 3 kDa fraction. The reducing power of bioactive peptides can be affected by the leucine and hydroxyproline contents. In a study performed by Ktari et al. (2020) showed the opposite results when zebra blenny (*Salaria basilisca*) protein hydrolysates were fractionated by ultrafiltration units with molecular weight cut-offs of 30, 10, 5, and 1 kDa. The results of their study revealed that the 10 < F < 30 kDa fraction had the highest antioxidant activities, including ferric reduction, DPPH radical scavenging, and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical scavenging. The amino acid composition can play an important role in reducing power of the peptide fractions for instance, the higher contents of glutamic acid, aspartic acid, glycine, and threonine and lower amounts of lysine can result in higher reducing activity (Paiva et al., 2017) which is in agreement with the results of the present study.

3.4. In vitro gastrointestinal digestion

The results of the *in vitro* gastrointestinal digestion effects on the

biological activities of fractions are shown in Fig. 2. The peptide concentration (PC) of different fractions was irregularly altered, with no trend observed. As shown in Fig. 2a, the peptide concentration of all fractions increased without statistical significance ($P < 0.05$).

The antioxidant activities of all three fractions were significantly decreased (Fig. 2b–f). The IC_{50} of the 3 < F < 10 kDa fraction, which had the highest DPPH radical scavenging (0.64 mg/mL) increased to 2.67 mg/mL. This was due to the digestion of 3–10 kDa peptides with high activity to lower molecular weight (< 3 kDa) peptides. The IC_{50} values of hydroxyl radical scavenging, superoxide radical scavenging, and ferrous chelation of the F < 3 kDa fraction, which showed the highest activity (0.88, 1.04, and 0.50 mg/mL) were significantly ($P < 0.05$) increased to 1.56, 2.63, and 1.03 mg/mL, respectively. The ferric reducing power of the F < 3 kDa fraction decreased from 0.58 to 0.24, after digestion. This reduction may be due to breaking down some peptides with ferric reducing power presented in the fraction. A study performed by Thuanthong, De Gobba, Sirinupong, Youravong, & Otte (2017) reported a reduction in activity of peptides after gastrointestinal effect which was in agreement with the present study.

Antibacterial activity was not an exception. These results (Fig. 2g) indicated that the MIC of the most active fraction (< 3 kDa) was significantly increased from ≤ 0.5 to 2.66 mg/mL. Similar results are reported by other studies (Xiao, Toldrá, Zhou, Gallego, Zhao, & Mora, 2020). In contrast, some studies have revealed different results indicating that the bioactivity of peptides has increased or remained unaffected after *in vitro* gastrointestinal digestion (Pimentel et al., 2020).

After drinking a beverage containing bioactive peptides, the structure and functionality of the peptides can be affected as they move through the gastrointestinal tract; specific alterations include hydrolysis, structural rearrangements, and aggregation when exposed to gastrointestinal tract fluids (Perry and McClements, 2020).

3.5. Antibacterial activity

The antibacterial activity of different fractions of the gelatin peptides against *E. coli* ATCC 8739 is shown in Fig. 3. The change in resazurin color from blue (oxidized state) to pink (reduced) is an index of microbial growth. The antibacterial activity of the F < 3 kDa fraction (MIC ≤ 0.50 mg/mL) was higher than that of other fractions. For the 3 < F < 10 and 10 < F < 30 kDa fractions, the MIC values were 4.00 and 8.66 mg/mL, respectively. For the F < 3 kDa fraction, the color of all three replicates did not change, indicating the potency of this fraction against *E. coli* cells, even at concentrations lower than 0.50 mg/mL which means the MIC was lower than this concentration.

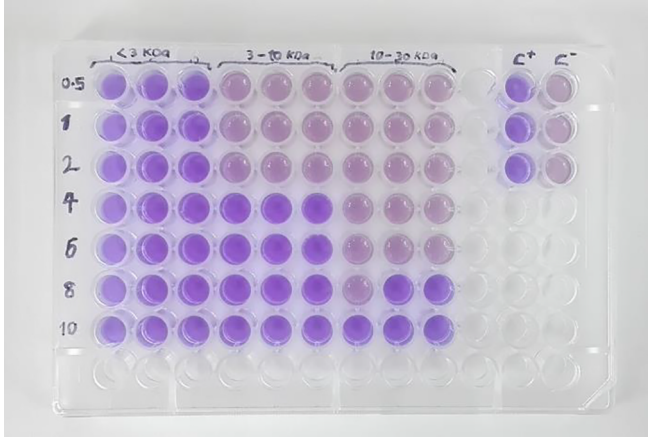


Fig. 3. The antibacterial activity of the different UF fractions of gelatin peptides. C^+ and C^- are positive (gentamycin at 20 μg/mL) and negative (sterile Mueller Hinton broth) controls, respectively.

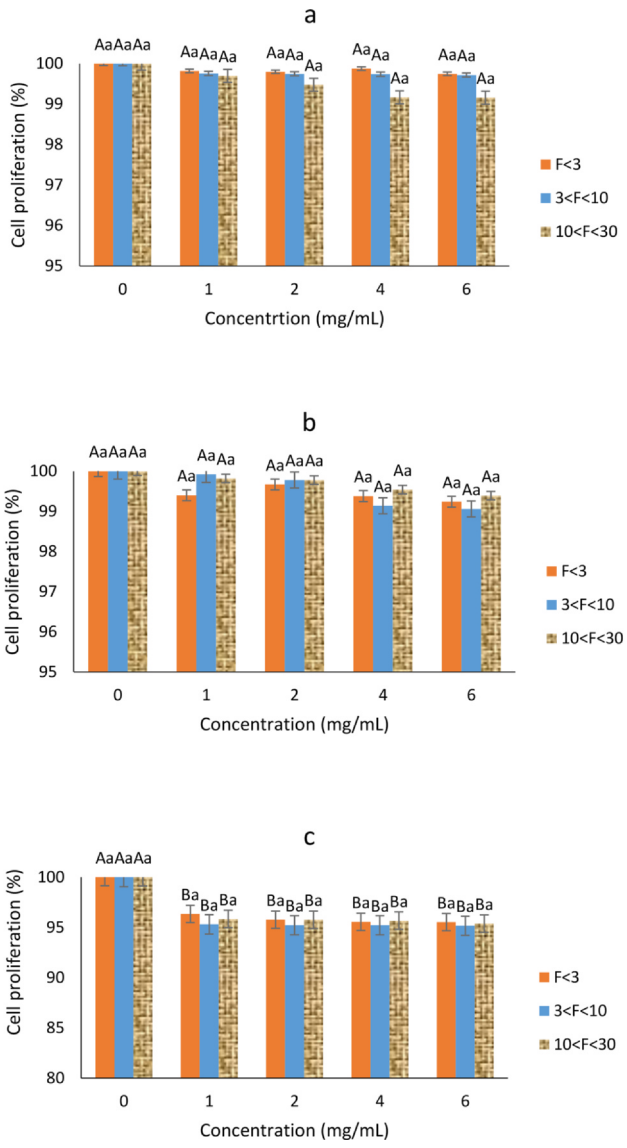


Fig 4. Cytotoxicity of different fractions at various concentrations. L929 (a), RAW264.7 (b), and Caco-2 cell lines (c). Different uppercase and lowercase leters represent the significant difference among various concentrations at the same fraction and various fractions at the same concentrations, repectively.

In a previous study, Pezeshk, Ojagh, Rezaei, & Shabanpour (2019) illustrated that the antibacterial activity of the lowermost molecular weight fraction (F < 3 kDa) of fish waste protein hydrolysates against both Gram-positive and negative bacteria was significantly higher ($P < 0.05$) than that of other fractions tested.

Interactions between peptides and the microbial cell membrane are key to antibacterial function. Anionic antibacterial peptides use metal ions, such as zinc, to generate cationic salt bridges with negatively charged structures in the target cell membrane; however, the antibacterial mechanism of these negatively charged peptides is not clearly elucidated (Harris, Dennison, & Phoenix, 2009).

3.6. Cytotoxicity

The results of cell proliferation assays for each cell line are depicted in Fig. 4. It was concluded that the treatment of cell lines did not affect cell proliferation compared to the control (with no sample). Although there was a slight decrease in cell proliferation after the addition of peptides, no significant difference was observed compared to the 100%

cell proliferation of the control ($P < 0.05$). The Caco-2 cell line showed a lower proliferation after addition of samples, but the other cell lines had a proliferation above 99%. The lowest cell proliferation of the Caco-2 cell line was 95.27%, which was related to the high peptide concentration (6 mg/mL). Notably, statistical analysis showed no significant difference among cell lines treated with different concentrations and the control. In our previous study (Mirzapour-Kouhdasht, Moosavi-Nasab, Krishnaswamy, & Khalesi, 2020), the amino acid sequence of the 3 < F < 10 kDa fraction was determined and an *in silico* assay showed no toxicity for that fraction, which corresponds to the results of the present study. To the best of our knowledge, there are only limited studies in which the cytotoxicity of gelatin hydrolysates against normal cells has been investigated. Lee, Moon, Kim, Park, Ahn, & Paik (2017) showed that ovotransferrin hydrolysates had < 20% cytotoxicity against MRC-5 (human normal lung fibroblastic cells).

3.7. Functional properties for carbonated beverage fortification

3.7.1. Solubility

In the present study, the investigation of solubility was the first priority among all functional characterization tests, owing to the purpose of the application of peptides in beverages. Other studies have confirmed that solubility is one of the most important properties of proteins and peptides to be applied in food and pharmaceutical products. The results of this study revealed that gelatin had lower solubility compared to its hydrolysate fractions at all pH values of 2–9 (Fig S1). The lowest solubility of gelatin (25.18%) was observed at a pH of 5, while before and after this point, the solubility increased. This was due to the alkaline pretreatment of gelatin leading to the formation of gelatin type B. The isoelectric point of gelatin type B is between the pH values of 4.7 and 5.2, and it is known that the lowest solubility of organic polymers will be achieved at the isoelectric point. Among peptide fractions, the F < 3 kDa fraction showed the highest solubility at all pH values, followed by the 3–10 and 10–30 kDa fractions, respectively. However, the highest solubility of the F < 3 kDa fraction (89.08%) was observed at a pH value of 2. The solubility of this fraction gradually decreased until pH 8, at which the lowest solubility (68.85%) was observed; with an increase in pH, the solubility increased again. This can be due to the saturation of the peptide surface with hydroxyl groups; thus, the addition of more hydroxyl groups creates a repulsion interaction and impedes the aggregation of the peptides (Wang and Zhang, 2012). For the other fractions, the same trend was observed. The lowest solubility (60.15% and 50% for the 3–10 and 10–30 kDa fractions, respectively) was observed at pH 8, and after an increase in pH above 8, the solubility increased again. The higher solubility at acidic pH values, could be a result of higher numbers of positively charged amino acids. Thus, at higher pH values, the positive charges on the peptide surface could be neutralized by electrostatic interactions with negatively charged hydroxyl groups, lowering the solubility (Wang and Zhang, 2012).

3.7.2. Water holding and fat-binding capacities

The evaluation of WHC and FBC is very important for proteins and peptides, especially those used in an aqueous solution such as a carbonated beverage. The WHC and FBC are dependent on the hydrophilic and hydrophobic amino acid contents (Kouhdasht, Moosavi-Nasab, & Aminlari, 2018). The results of this experiment (Table 2) indicated that the WHC of the F < 3 kDa fraction (109.22%) was significantly ($P < 0.05$) higher than that of the other fractions. The WHC of the 3 < F < 10 kDa fraction (97.13%) did not significantly differ from that of the 10 < F < 30 kDa fraction (96.04%), which was probably due to the approximately equal content of hydrophilic amino acids (as shown in Table 1). Le Vo et al. (2020) have reported results agreeing with the present study. The authors stated that the lower molecular weight peptides derived from *Acetes japonicus* protein hydrolysates had a higher WHC than those with higher molecular weights due to higher hydrophilicity (hydrophilic amino acids).

A higher FBC was observed in the F < 3 kDa fraction (98.05%) compared to other fractions, which was related to the hydrophobic amino acid content. This dual character of the F < 3 kDa fraction could be an advantage and act as emulsifiers such as phospholipids. The experiments of Le Vo et al. (2020) revealed that the FBC of the *Acetes japonicus* protein hydrolysates was 1.59 mL oil per gram. In addition to hydrophobic amino acid content, the surface hydrophobicity, bulk density, and enzyme-substrate specificity affects the FBC of proteins and peptides (dos Santos, Martins, Salas-Mellado, & Prentice, 2011).

3.7.3. Emulsion activity and stability

Two of the major functional properties of proteins and peptides are their emulsion activity and stability. The results of these two criteria are shown in Table 2. The molecular weight is inversely correlated with the EAI value. The F < 3 kDa fraction, with EAI and ESI values of 157.20 m^2/g and 29.25 min, was indicated to be an ideal fraction for use in carbonated beverages. The EAI of the 3 < F < 10 kDa fraction (118.35 m^2/g) was significantly ($P < 0.05$) higher than that of the 10 < F < 30 kDa fraction (99.05 m^2/g), while the ESI was not significantly different (37.40 and 38.73 min, respectively). The higher EAI is indicative of finer and more properly dispersed oil globules; Hajfathalian, Ghelichi, García-Moreno, Moltke Sørensen, & Jacobsen (2018) have demonstrated that the lower molecular weight peptides have a higher EAI, which may be due to finer and more properly dispersed oil globules. This feature induces the unfolding and rearrangement of the molecule to diffuse at the oil–water interface.

The lower ESI of the F < 3 kDa fraction was probably due to the incapability of the lower molecular weight molecules to form a film around droplets at the oil–water interface.

3.7.4. Foam expansion and stability

The differences in FE and FS of different fractions are shown in Table 2. The results of the present study revealed that the FE of the F < 3 kDa fraction (117.32%) was significantly lower than that of the 3–10 kDa and 10–30 kDa fractions (161.18 and 210.50%, respectively). Similar results have been obtained from other studies (Zamorano-Apodaca et al., 2020). The higher the molecular weight, the more the FE increases. The most reasonable explanation for this result is the increased ability of the higher molecular weight molecules to rearrange their structure at the oil–water interface (Hajfathalian, Ghelichi, García-Moreno, Moltke Sørensen, & Jacobsen, 2018). The FS of all three fractions was significantly ($P < 0.05$) decreased after 30 and 60 min. The FS of the F < 3 kDa fraction after 30 and 60 min was lower than that of the other fractions at the same time. It appears that the similar reason for the ESI can be applied to FS, except that the inability of the lower molecular weight peptides to form a viscose film around air cells is the reason for their lower FS. Identical results have been reported in several studies in which the FS of gelatin hydrolysates was determined (Zamorano-Apodaca et al., 2020).

3.7.5. Gushing test

Overfoaming is an important parameter in carbonated beverages due to its effect on customer satisfaction. The term gushing is related to the uncontrolled discharge of CO_2 following the opening of a bottle, leading to the beverage/foam gushing out. There are two types of gushing: the first is related to the interaction of fungal proteins called hydrophobin and CO_2, while the second is related to other factors such as supersaturation of CO_2, heavy metals, and the rough surface of the bottle (Hégrová et al., 2009). The results of the gushing test are shown in Table 2. The overfoaming was positively related to the FE of peptides. Similarly, Stanislava (2007) reported that overfoaming, FE, and antibacterial peptides in beer were inter-related. The lowest amount of overfoaming (6.93%) corresponded to the F < 3 kDa fraction, and its value was significantly ($P < 0.05$) lower than that of the other fractions. This analysis revealed that the lower the molecular weight, the

Table 2
The functional properties of different fractions of peptides.

Fractions	WHC (%)	FBC (%)	EAI (m^2/g)	ESI (min)	Over foaming (%)	FE (%)	FS (%)	
							30 min	60 min
F < 3	109.22 ± 1.01^{a}	98.05 ± 1.45^{a}	157.20 ± 1.41^{a}	29.25 ± 0.25^{b}	6.93 ± 0.32^{c}	117.32 ± 1.13^{Ac}	86.11 ± 0.45^{Bc}	75.03 ± 0.52^{Cc}
3 < F < 10	97.13 ± 1.73^{b}	79.84 ± 0.81^{b}	118.35 ± 1.11^{b}	37.40 ± 0.71^{a}	9.03 ± 0.24^{b}	161.18 ± 1.25^{Ab}	120.75 ± 1.00^{Bb}	104.20 ± 1.43^{Cb}
10 < F < 30	96.04 ± 1.15^{b}	67.26 ± 0.59^{c}	99.05 ± 1.36^{c}	38.73 ± 1.00^{a}	11.25 ± 0.81^{a}	210.50 ± 0.95^{Aa}	194.62 ± 1.78^{Ba}	161.05 ± 1.88^{Ca}

Data are shown as mean ± SD of three replicates. Different superscript letters in the same column represent the significant difference ($P < 0.05$). Different uppercase letters in FE and FS data, represent the significance among rows ($P < 0.05$).

less overfoaming occurred. Since overfoaming is one of the general problems that occurs in carbonated beverage industries, it can be concluded based on the results that the F < 3 kDa fraction is the most appropriate to use.

To the best of our knowledge, no study has determined the effect of exogenous bioactive peptides on non-alcoholic carbonated beverage gushing.

3.7.6. Flavor evaluation of hydrolysates

To prepare a new product derived from marine sources, the fishy flavor, which may persist and lower the acceptability, has to be determined. The panelists revealed that the flavor of the F < 3 kDa fraction was desirable at all concentrations up to 2 mg/mL. The scores of the 3 < F < 10 kDa fraction showed that this sample had a threshold fishy flavor at the concentration of 1.5 mg/mL, and the fishy flavor could be sensed slightly at a concentration of 2 mg/mL. The 10 < F < 30 kDa fraction showed a slightly fishy flavor even at the lowest concentration (1 mg/mL); at the concentrations of 1.5 and 2 mg/mL, the scores of this sample were 4.00 and 4.15, respectively. These results indicated that the 10 < F < 30 kDa fraction had a significantly fishy flavor compared to the other fractions (Table S-1). To the best of our knowledge, no prior study has evaluated the fishy flavor of fractionated peptides.

3.7.7. Color

The results of the color characterization of different fractions of peptides are shown in Fig S2. The L* value is a prominent factor in the functional properties of peptides due to its application in beverages. The L* values of the F < 3 kDa, 3–10 kDa, and 10–30 kDa fractions were 43.13, 39.25, and 36.80, respectively. The differences among all three fractions were significantly different, indicating that the F < 3 kDa fraction was lighter than the other fractions and appropriate for application in beverages without changing the lightness. The a* values of fractions in the abovementioned order were −19.51, −15.45, and −10.26, respectively. Finally, the b* values of the different fractions were 24.33, 18.51, and 13.10, respectively. These results showed that ultrafiltration had a significant effect on color factors that could be a result of the concentration of pigments during filtration.

4. Conclusion

The present study revealed that different fractions of fish gelatin hydrolysates obtained by a sequential enzymatic procedure (actinidin-Alcalase®) could be used for the enrichment of carbonated beverages. The investigation of biological activities, such as antioxidant, antibacterial, and functional properties, including solubility, water and fat-binding, emulsifying activity, foaming characteristics, overfoaming, color, and flavor, along with non-toxicity, indicated that the F < 3 kDa fraction could be a promising ingredient to fortify carbonated beverages. Designing an appropriate delivery system for these bioactive peptides to decrease the negative effects of gastrointestinal digestion and also promote their absorption by epithelial cells could be a subject for future studies.

CRediT authorship contribution statement

Armin Mirzapour-Kouhdasht: Conceptualization, Investigation, Methodology, Software, Supervision, Visualization, Writing - original draft. **Marzieh Moosavi-Nasab:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Supervision, Validation, Writing - review & editing. **Young-Min Kim:** Data curation, Formal analysis, Funding acquisition. **Jong-Bang Eun:** Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was financially supported by Shiraz University, Iran [Grant number: GR-AGR-56], the Heilongjiang Provincial Natural Science Foundation of China [grant no. ZD2019C002], and the National Key Research and Development Program of China [grant no. 2018YFD0401101].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.128339.

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