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Bioactive and Functional properties of gelatin peptide fractions obtained from sea bass (*Dicentrarchus labrax*) skin

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Abstract

Fish skin is one of the most common resources of gelatin, which can be hydrolyzed bioactive peptides. In this study, gelatin from sea bass skin (SBS) was hydrolyzed with flavourzyme® to obtain peptide fractions with different molecular weights and determined their bioactive and functional properties. All peptide fractions obtained showed antioxidant activity (DPPH and FRAP). The bass gelatin peptide fraction 1 (BF1) (≤5 kDa) showed the highest DPPH (44.9%) and FRAP (42.04 mmol Fe^{+2} /g and 22.98 mmol trolox/g) activities. Besides, the BF1 (≤5 kDa) peptide fraction showed the highest *in vitro* cytotoxic effect (16.58%) at 20 mg/mL concentration compared to the other peptide fractions. The highest emulsifying capacity (389.5 m^2/g), emulsifying stability (53.2 min), foaming capacity (30.47%), and foaming stability (10.40%) were obtained from the control gelatin sample. Moreover, the BF3 (≥10 kDa) peptide fraction showed an excellent fat binding capacity (9.39 mL/g). Enzymatic hydrolysis decreased emulsifying and foaming capacity of gelatin while increasing its fat binding capacity. In particular, antioxidant and anticancer activities of peptide fractions with low molecular weight were found to be high. The results demonstrated that gelatin and hydrolysates from the SBS offer an important alternative as a functional food ingredient for food technology.

Keywords: sea bass skin; gelatin peptide fractions; bioactive peptides; *in-vitro* cytotoxic activity; functional properties.

Practical Application: Bioconservation of fish skin waste into valuable product has highly potential to reduce pollution and industrial cost and thus, to provide economic development. Peptides with different molecular weights are produced from fish waste by enzymatic hydrolysis and can be concentrated with ultrafiltration membrane. The low molecular weight peptides obtained can be used as functional food agents, while the high molecular weight ones can be used to improve technological properties in the food industry.

1 Introduction

Global fish production is estimated to reach approximately 179 million tons in 2018 and 204 million tons in 2030; however, only 156 million tons of this amount is suitable for human consumption (Food and Agriculture Organization of the United Nations, 2020). Depending on the species and processing level, 20% to 80% of fish waste is derived from fish for human consumption. More than 50% of these wastes are provided as heavy wastes, which are not suitable for human consumption, with a very high risk of environmental pollution (Yathisha et al., 2019). However, it is also possible to produce products with high added value such as oil, collagen, gelatin, hydrolysate and bioactive peptide production from fish wastes (Ishak & Sarbon, 2017; Ishak & Sarbon, 2018).

During the production of fish products such as fillets, the wastes, including the wastes derived from the skin, bones, and scales, contain raw materials rich in collagen up to 30%. Gelatin is produced by thermal denaturation of collagen, which is the main protein of fish skin. Gelatin is used in the food industry to improve elasticity, consistency, and stability and an important agent in the pharmaceutical industry in terms of encapsulation and film forming process (Gómez-Guillén et al., 2002). In addition, gelatin is hydrolyzed to be used as a source of bioactive peptides. Hydrolysates can be produced using fish waste through enzymatic hydrolysis, autolysis, and thermal hydrolysis. Enzymatic hydrolysis is the most common and reliable one among these methods (Ishak & Sarbon, 2018).

Enzymatic hydrolysis is an effective method to convert low-consumption proteins into high-value products and to improve their biological activity and nutritional value (Rajabzadeh et al., 2018). pH, temperature, enzyme/substrate ratio, hydrolysis time, enzyme type and substrate are important factors in the enzymatic hydrolysis process (Bozkurt et al., 2021). In particular, the selected enzyme and substrate is a critical factor that affects both the properties and composition of the hydrolysate, the amino acid sequence of the obtained peptides, and the functional properties (Fan et al., 2018). Protein hydrolysis confers modified functional properties such as solubility, water and oil binding, emulsification and foaming compared to the parent protein (Bozkurt et al., 2021).

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Bioactive peptides are protein fragments with low molecular weight, containing 2-20 amino acids, formed by the enzymatic hydrolysis of proteins, with some physiological health benefits to the body (Halim et al., 2016). Molecular weight, hydrophobicity, length and amino acid sequence, surface charge, and secondary structure determine the bioactive properties of peptides (Roudi et al., 2017). In general, peptides with high molecular weight have higher technological properties, while peptides with low molecular weight have higher bioactive properties (Ishak & Sarbon, 2018). Low molecular weight peptides obtained from gelatin of fish by-product origin by enzymatic hydrolysis, have functional properties such as water solubility, emulsification, and foaming, and fat binding, as well as bioactive properties such as antioxidant, anticancer, antibacterial, antimicrobial, antihypertensive (ACE inhibitor), anti-hyperglycemic and anti-Alzheimer's features (Abuine et al., 2019; Ishak & Sarbon, 2018; Mirzapour-Kouhdasht et al., 2020; Tkaczewska et al., 2020; Zamorano-Apodaca et al., 2020).

Ultrafiltration, gel filtration, ion exchange chromatography, and reverse phase high-performance liquid chromatography (RP-HPLC) techniques are used to purify peptides from protein hydrolysates (Ishak & Sarbon, 2018). Membrane filters with different molecular weight separation in the range of 1-10 kDa are used in ultrafiltration, one of the purification methods, and the peptide fractions obtained by the sequential ultrafiltration technique have a purity between 1.51 and 525 times higher than the main hydrolysate (Ishak & Sarbon, 2018).

In Turkey, which is the most significant the sea bass (*Dicentrarchus labrax*) producer globally, 137,419 tons of sea bass was produced in 2019 (Turkish Statistical Institute, 2020). However, studies on the utilization of sea bass wastes are almost nonexistent. Surprisingly, there are only a few studies on production of protein hydrolysate from the wastes of the sea bass (Altınelataman et al., 2019; Erol et al., 2017; Valcarcel et al., 2020; Yilmaz et al., 2016). Most likely this is the first study focus on production of gelatin hydrolysate from the SBS and determination of peptide fractions and its anticancer and antioxidant properties.

In the present study, it was aimed to determine the bioactive (antioxidant and cytotoxic) and functional (emulsification, foaming, and fat binding) properties of three peptide fractions produced from the gelatin hydrolysates of the sea bass *(Dicentrarchus labrax)* skin origin.

2 Materials and methods

2.1 Materials and chemicals

Flavourzyme® (protease from *Aspergillus oryzae*), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). Polyethersulfone membranes were purchased from Sartorius Stedim Biotech GmbH (Göttingen, Germany). All other chemicals and solvents were analytical grade.

2.2 Preparation of fish skins

SBS (average weight of sea bass: 350 ± 20 g) were obtained from Kilic Deniz Inc. (Mugla/Turkey). After delivering to the laboratory by maintaining the cold chain, impurities such as scales and meat residues found on the skins were removed with a knife immediately. The skins were then washed with cold tap water and drained. The cleaned skins were maintained in the freezer at -20 °C (maximum two months) until processed. The skins used in gelatin production were thawed in the refrigerator at 4 °C and cut approximately 2 - 3 cm^2 in size.

Production of gelatin from fish skins

Gelatin production from fish skins was performed according to the method described by Boran & Regenstein (2009). Briefly, the fish skins were placed on a 500 mL conical flask and treated first with NaOH (5:1 v/w, 0.55 N, 67.5 min) alkaline solution, followed by HCl treatment (5:1 v/w, 0.1 N, 45 min) acid solution. After each alkaline and acid treatment, the skins were washed with distilled water (5:1 v/w) at room temperature, filtered using a 4-layer sterile cheesecloth, and were squeezed with hand. Subsequently, extraction was performed using pure water (4:1 v/w, 50 °C, 3 hr) in a water bath (Memmert WNB45, Schwabach, Germany). Following the extraction, fish skins were removed by filtering through a 4-layer cheesecloth. Gelatin solutions were placed in glass containers and dried in an oven (Binder GmbH ED240, Tuttlingen, Germany) at 60 °C for approximately 72 hours. Gelatin was obtained in the form of leaves and stored in dry conditions in polyethylene packages until use.

2.3 Production of gelatin hydrolysate

Firstly, 5% (w/v) of gelatin solution was prepared using purified water and stirred gently overnight at ambient temperature for complete dissolution. Then, pH degree of solution (Mettler Toledo, S220, Switzerland) was adjusted to 7.0 with a 0.1 M NaOH solution, and hydrolysis was carried out using flavourzyme® (E/S: 1/100 [v/p]) at 50 °C for 1 hour. Next, the enzyme was inactivated by heating at 90 °C for 20 minutes. The solutions were centrifuged (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 10.000 rpm for 15 minutes. They were allowed to dry at -50 °C in a freeze dryer (Martin Christ GmbH, Beta 1-8 LSCplus, Osterode am Harz, Germany). Hydrolysates, which were in the form of dry powder, were packaged and stored for further analyses.

2.4 Determination of the degree for hydrolysis

The pH-stat method was used to determine the degree of hydrolysis (Blanco et al., 2017). The pH-stat is used to keep the pH value constant, which changes due to hydrogen ions released or consumed during the reaction. The degree of hydrolysis is defined as the proportion of the cleaved peptide bonds (h) to the total number of bonds per unit weight (h_{tot}). The following Equation 1 was used to calculate the degree of hydrolysis:

$$\%DH = \frac{hx100}{h_{tot}} = \frac{BxN_b x100}{\alpha x M_p x h_{tot}} \quad (1)$$

B: base amount,

N_b: base normality,

α: mean dissociation constant of the α-NH_2 groups,

M_p: the amount of protein (g),

h_{tot} means total peptide bonds (meqv/g protein) = 8.41 mmol/g protein (Zhang et al., 2012).

2.5 Obtaining gelatin peptide fractions

Peptide fractions from gelatin hydrolysates were obtained by sequential ultrafiltration. Polyethersulfone membranes were used for separating into molecular weights of 5 and 10 kDa. As a result of the ultrafiltration process (Sterlitech HP4750, Washington, USA), three peptide fractions were obtained as bass gelatin peptide fraction 1 (BF1) (≤5 kDa), bass gelatin peptide fraction 2 (BF2) (5-10 kDa), and bass gelatin peptide fraction 3 (BF3) (≥10 kDa). The gelatin peptide fractions were immediately lyophilized (Zamorano-Apodaca et al., 2020).

2.6 Fourier Transform Infrared (FTIR) spectroscopy analysis

Both of the 10% solutions of gelatin (w/v) and peptide fractions (w/v) were prepared using pure water. The solutions were kept in a water bath (Memmert WNB45, Schwabach, Germany) at 45 °C for 30 minutes to obtain complete homogenization. All the measurements of ATR-FTIR spectrums of the solutions were performed at 4 cm^{-1} resolution and in the form of 16 scans per spectrum (Bruker Tensor 27 FTIR Spectrometer, Bremen, Germany). The spectrums were recorded in 4000-600 cm^{-1} mid-infrared areas. The mean of the three spectra measured under the same conditions was calculated for each sample. Before each measurement, the crystal surface was cleaned with pure water and 100% pure ethyl alcohol (Cebi et al., 2016).

2.7 Zeta potential values of gelatin peptide fractions

The 10% solutions of gelatin (w/v) and peptide fractions (w/v) were prepared using pure water. The samples were kept in a water bath (Memmert WNB45, Schwabach, Germany) at 45 °C for 30 minutes to obtain complete dissolution. The sample of 25 µL was mixed with 2 mL of PBS (0.01 M and pH 7.4), and the measurement was performed using the Zetasizer Nano ZSP (Malvern, United Kingdom) device. Zeta potential values were obtained as means from 3 processes involving a minimum of 15 measurements (Bozkurt et al., 2021).

2.8 Determination of functional properties

Emulsifying activity and stability

The emulsifying activity index (EAI) and emulsifying stability index (ESI) were determined with the slightly modified version of the method mentioned by Zamorano-Apodaca et al. (2020). A total of 30 mL was extracted from the lyophilized gelatin peptide fraction solutions (0.1% p/v), and 3 mL of corn oil was added. The mixture was homogenized for 1 minute using the Ultra-turax (Daihan HG-15D, Seoul, Korea) device at 20.000 x *g*. After the homogenization, the emulsification obtained from the bottom part of the emulsification at the end of 0 and 10 minutes was diluted to 5 mL using 0.1% (w/v) dodecyl sulphate sodium salt (SDS). Absorbance values of the samples were measured at 500 nm wavelength using a spectrophotometer (Shimadzu, UV-1800, Tokyo, Japan). The readings of the absorbance values (A_0 and A_{10}), which were performed for calculating the EAI and ESI values, were used in the following equations (Equation 2 and 3):

$$EAI\ \left(m^2/g\right) = \left(2\ x\ 2.303\ x\ A_0\right)/\ (C\ x\varphi) \quad (2)$$

A_0 = Absorbance at 500 nm wavelength

C = Initial protein concentration (g/mL)

φ = Oil volume in emulsification

$$ESI\ (min) = (A_0 x \Delta t)/\Delta A \quad (3)$$

A_{10}= Absorbance at the end of 10 minutes

Δt = 10 min

ΔA = A_0 - A_{10}

Foaming capacity and stability

The foaming capacity and foaming stability were determined with slight modifications to the method mentioned by Zamorano-Apodaca et al. (2020). A total of 10 mL was extracted from the lyophilized gelatin peptide fraction solutions (0.5% p/v), and it was homogenized for 2 minutes using the Ultra-turax (Daihan HG-15D, Seoul, Korea) device at 16.000 x *g*. The total foaming volume was measured after 0, 3, and 10 min due to homogenization. Foaming capacity referred to the foaming expansion that occurred at 0 min while foaming stability referred to the foaming expansion that occurred at 10 min. The foaming expansion was calculated using the following Equation 4:

$$Foaming\ expansion\ (\%) = \left[(A-B)/B\right] x100 \quad (4)$$

A: Volume at different times (mL)

B: Volume before homogenization (mL)

Fat binding capacity

Fat binding capacities of the peptides were determined with slight modifications to the method described by Karoud et al. (2019). A sample of 20 mg was placed into the tared centrifuge tubes and weighed. After adding 400 µL of corn oil, it was maintained at room temperature for 1 hour. The mixture was mixed thoroughly by vortex (Velp ZX3 Vortex Mixer, Usmate (MB), Italy), mixing for 5 seconds every 15 minutes. The solutions were centrifuged (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) at 5000 rpm for 10 minutes, and the fat base was removed. For the control group, oil was added to

an empty centrifuge tube without placing the sample. The fat binding capacity was expressed as mL oil/g protein.

2.9 Antioxidant properties of gelatin hydrolysates

Antiradical activity by 2,2-Diphenyl-1-Picryl Hydrazyl (DPPH) method

DPPH free radical scavenging activities of the gelatin peptide fractions were determined with a slightly modified method of Dara et al. (2020). 1.5 mL of solutions (20 mg/mL) prepared from gelatine hydrolysate fractions was taken and 1.5 mL of 0.2 mM DPPH solution prepared in methanol was added. The mixture was mixed thoroughly by vortex (Velp ZX3 Vortex Mixer, Usmate (MB), Italy) at high speed. The solution was maintained at room temperature in the dark for 20 minutes. Pure water was used instead of the solution in the control samples. The absorbances of the samples were measured at 517 nm wavelength using a spectrophotometer (Shimadzu, UV-1800, Tokyo, Japan). A low absorbance value indicated high radical scavenging activity. The DPPH radical scavenging activity was calculated using the following Equation 5:

$$DPPH\ free\ radical\ scavenging\ activity\ (\%) = \left[1 - Abs_{sample} / Abs_{control}\right] x100 \quad (5)$$

Ferric Reducing Antioxidant Power (FRAP) method

The antioxidant potential of peptide fractions was determined according to the method of Ketnawa et al. (2017) with slight modifications. The oxidant used in the FRAP analysis consisted of the acetate buffer (pH 3.6), iron chloride solution (20 mM), and 2,4,6-Tris (2-pyridyl)-s-triazine solution (10 mM TPTZ in 40 mM HCl). It was freshly prepared on the day of the analysis and used at the proportion of 1:1:10 (v/v/v), respectively. 1 mL of solutions (20 mg/mL) prepared from gelatine hydrolysate fractions was taken and 2 mL of FRAP solution was added on it and vortexed. The tubes were maintained at room temperature for 30 minutes in the dark, and the measurements were performed using a spectrophotometer (Shimadzu, UV-1800, Tokyo, Japan) at a wavelength of 593 nm. The FRAP analysis results were calculated by drawing a calibration curve of $FeSO_4.7H_2O$ and trolox as mmol Fe^{+2} and mmol trolox equivalence value at g hydrolysate.

2.10 Determination of in-vitro cytotoxic activity

The *in vitro* cytotoxicity activity of the samples was performed using the XTT method described by Cakir-Koc et al. (2018) with some modifications. Caco-2 adenocarcinoma cell line was used for the cytotoxic effects of samples. In brief, cell line was incubated at 37 °C for 3-4 days in a 5% CO_2 incubator. After incubation, the cells were removed by a trypsinization process and counted with Thoma cell counting chamber. Later, the cells were added on a 96-well microplate as 10^4 cell/well, and they were incubated at 37 °C for 24 hours for the cells to attach to the bottom of the wells. After the incubation, the samples in different concentrations were added into the wells and were incubated again for 24 hours at 37 °C in an incubator containing 5% CO_2. Subsequently, the medium parts of the wells were removed and the 100 µL 2,3-bis-(2-methoxy-4-nitro-5-sul-fophenyl)-2H-tetrazolium-5-carboxanili-de (XTT, 0.5 mg/mL) solution was added into the wells. The microplates were incubated at 37 °C for 3 hours. At the end of the incubation, the optical density (OD) was measured by a microplate reader (Biotek, Elx800, VT, USA) at 450 nm wavelength. The number of viable cells was calculated according to the following formula (Equation 6):

$$Viability\ (\%) = (OD\ value\ of\ samples\ /\ OD\ value\ of\ controls)\ x\ 100 \quad (6)$$

2.11 Statistical analysis

Analyses were run in triplicate and results were reported as mean values ± standard deviation (S.D). Data were subjected to analysis of variance (one-way ANOVA) The statistical analysis was performed using JMP 9 software.

3 Results and discussion

3.1 Degree of hydrolysis

The degree of hydrolysis is a parameter used to monitor proteolysis and to estimate the size of peptides formed as a result of enzymatic hydrolysis. Parameters such as pH value, temperature, time and enzyme concentration affect the degree of hydrolysis significantly. In this study, due to the high rate of hydrolysis, the degree of hydrolysis reached 2% in the first 20 minutes of hydrolysis, and then the hydrolysis rate started to decrease and reached 3.6% at the end of 60 minutes (Supplementary). This reduction in hydrolysis rate over time is related to the depletion of the substrate and the potentially negative impact of reaction products that can saturate the enzyme's active site and inhibit its catalytic mechanisms. In a similar research conducted by García Arteaga et al. (2020) the hydrolysis degree of pea protein was determined as 3.88% at the end of the flavourzyme® induced hydrolyzation period of 60 min. Similarly, about 4% was obtained for the hydrolysates derived from silver carp muscles using flavourzyme® (Dong et al., 2008). The degree of hydrolysis and the molecular weight of the peptides formed depend on the type of enzyme used, and as the degree of hydrolysis increases, the bioactive and functional properties of the formed peptide fractions also change. Flavourzyme is a mixed endo- and exopeptidase enzyme produced from *Aspergillus oryzae* (Bozkurt et al., 2021). As with every enzyme, flavourzyme has a different specificity and separates the polypeptide chains from different parts (Kchaou et al., 2020). Therefore, it provides a different degree of hydrolysis, which is considered a distinguishing feature among hydrolysates (Baharuddin, 2016). The degree of hydrolysis affects the bioactive and functional properties of gelatin hydrolysates. A degree of hydrolysis below 10% yields peptides in more significant sizes, thereby improving their functional properties such as emulsification and foaming capacity (Arteaga et al., 2020; Mirzapour-Kouhdasht et al., 2021; Zamorano-Apodaca et al., 2020). In this study, the enzymatic hydrolysis process increased the oil binding capacity compared to the main protein gelatin, while decreasing the emulsion and foaming values (Figure 2, 3 and 4). This may be due to the partial hydrolysis of gelatin during its production from collagen. In addition, although the degree of

hydrolysis is low, the antioxidant capacity (DPPH and FRAP) values obtained in our study are quite high (Table 1).

3.2 Zeta potential values of gelatin peptide fractions

The zeta potential refers to the negative charge prevalence of the peptide fractions. The zeta potential values of the gelatin peptide fractions are displayed in Table 1. The most stable peptide fraction was the BF1 (-7.25 ± 1.17) peptide fraction with the highest zeta potential value. The sample with the lowest zeta potential value was gelatin from SBS. The enzymatic hydrolysis changes the protein charge and its electrokinetic mobility, as it causes decomposition in functional groups of amino acids. Various studies reported that the high value of zeta potential (in the "+" or "-" direction) caused an increasing repulsive interaction between the molecules, thereby decreasing the frequency of collision in molecules and improving the stability of the solution (Arias-Moscoso et al., 2015; Zamorano-Apodaca et al., 2020). It was reported that one of the most stable peptide fractions, which were obtained from collagen hydrolysates of 10 different fish waste mixtures, was the peptide fraction with the lowest molecular weight (<1 kDa) (Zamorano-Apodaca et al., 2020).

3.3 Fourier Transform Infrared (FTIR) spectroscopy analysis

The FTIR spectrums of gelatin from SBS and peptide fractions are displayed in Figure 1. FTIR spectroscopy is used to imaging the secondary structure and functional groups of gelatin (Nagarajan et al., 2012). The Amide I band was observed at 1622 - 1633 cm^{-1} wavelengths. Similar results were obtained by Nagarajan et al. (2012), and it was reported that the wavelength of 1633 cm^{-1} was characteristic for the coil structure of gelatin. C = O and CN stretching vibrations usually cause Amide I band, and absorption in this area helps determine the secondary structure of proteins by infrared spectroscopy (Cebi et al., 2016).

The Amide II band was found at 1539 - 1544 cm^{-1} wavelengths. Amide-II bands are caused by the N – H groups' bending vibrations and the C – N groups' strain vibrations. Similar results were also obtained by Nagarajan et al. (2012). The Amide III band was found at 1251-1249 cm^{-1} wavelengths. Amid III refers to the combination peaks between C – N strain vibrations and NH deformation resulting from the amid bands and the vibration stresses from the CH_2 groups (Jackson et al., 1995). The Amide B band was observed at 3064-3062 cm^{-1} wavelengths, corresponding to the asymmetric stretching vibration of = C–H as well as $–NH_3^+$. 1000-1100 cm^{-1} spectral area is caused by the vibration of the ν(C – O) and ν(C – O – C) bonds belonging to the carbohydrates. In particular, the 1083-1031 cm^{-1} range refers to the wavelength belonging to C-O strain vibrations of the carbohydrate residues found in the collagen, and it represents the Amide I in the proteoglycans (Kohler et al., 2007). In this study, the wavelengths of 1075-1066 cm^{-1} were identified for peptide fractions and gelatin. A similar result was emphasized

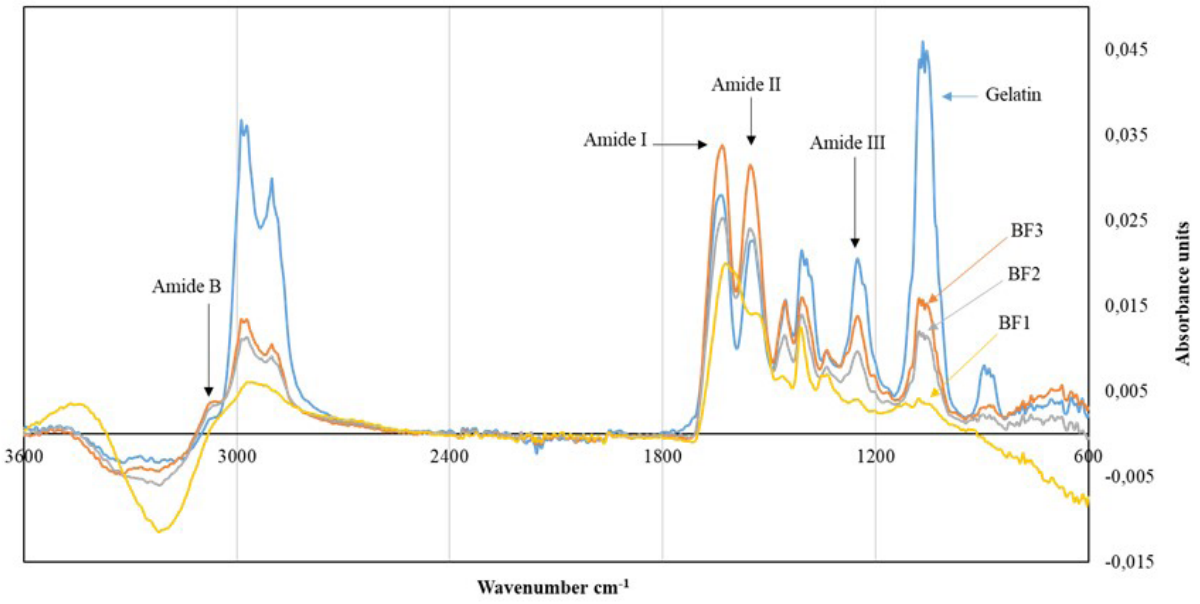

Figure 1. The FTIR (Fourier Transform Infrared) spectrums of gelatin from SBS and peptide fractions. BF1: ≤5 kDa, BF2: 5-10 kDa, BF3: ≥10 kDa.

Table 1. Zeta potential, FRAP (Ferric Reducing Antioxidant Power) and DPPH antioxidant capacity of the gelatin peptide fractions.

Sample	Zeta potential values (mV)	mmol Fe^{+2}/g hydrolysate	mmol trolox/g hydrolysate	DPPH activity (%)
BF1 (≤5 kDa)	-7.25 ± 1.17^{x}	42.04 ± 1.72^{a}	22.98 ± 0.94^{a}	44.9 ± 1.71^{A}
BF2 (5-10 kDa)	-5.37 ± 0.10^{xy}	23.68 ± 0.99^{b}	13.15 ± 0.54^{b}	29.32 ± 2,04^{B}
BF3 (≥10 kDa)	-3.66 ± 0.37^{y}	27.84 ± 0.62^{b}	15.37 ± 0.34^{b}	24.34 ± 0.02^{B}
Gelatin	2.59 ± 0.82^{z}	ND	ND	ND

Data are expressed as means ± SD from triplicate determinations. ND: Not Detected. Different letters indicate significant statistical differences between samples ($p < 0.05$).

by Cebi et al. (2016) in gelatin from cold water fish skin . Amide I, II, III and B absorbances of gelatin peptide fractions showed lower peaks compared to gelatin. Especially the BF1 peptide fraction gave the lowest peaks in the amide regions. This is probably due to secondary structure loss as a result of enzymatic hydrolysis (Guillén et al., 2010).

3.4 Functional properties

Emulsifying Activity (EAI) and Stability (ESI)

The EAI and ESI values of gelatin and peptide fractions are presented in Figure 2. The highest value obtained in EAI belonged to the gelatin from SBS (389.5 ± 15.9 m^2/g), which was followed by the BF3 (233.1 ± 12.6 m^2/g), BF2 (184.2 ± 1.81 m^2/g), and BF1 (79.33 ± 17.3 m^2/g) peptide fractions.

There was a decrease in the EAI value as the molecular weight decreased. Besides, enzymatic hydrolysis significantly reduces emulsion activity of gelatin produced by partial hydrolysis from the primary protein collagen (Figure 2). As in EAI, the highest value obtained in ESI belonged to the gelatin from SBS (53.22 ± 9.63 min), which was followed by the BF1 (38.8 ± 3.46 min), BF2 (31.6 ± 3.53 min), and BF3 (21.6 ± 2.32 min) peptide fractions. The ESI value increased as the molecular weights of the peptide fractions decreased, except in gelatin. The ESI value of gelatin was considerably higher compared to the peptide fractions.

The peptide fractions are lower than gelatin in both EAI and ESI values. This could be due to the partial hydrolyzation of gelatin during its production from collagen. Also, excessive amounts of peptides with high molecular weight or hydrophobic peptides contribute to emulsifying stability. Peptides with low molecular weight may not be amphiphilic enough to exhibit good emulsifying properties (Klompong et al., 2007).

Similarly, Alolod et al. (2019) argued that the emulsifying properties of gelatin hydrolysates from Oneknife Unicorfish skin were affected by molecular properties, such as the concentration and size of the peptides. They reported that the small peptides rapidly migrated to the oil-water interface; however, they could not stabilize the emulsification due to decreased hydrophobic/hydrophilic balance. Hydrolysates are surfactants, and they support oil-in-water emulsification due to their hydrophilic and hydrophobic groups and associated charges (Gbogouri et al., 2004). Besides the peptide's size, the peptides' amphiphilic nature is essential for the interfacial and emulsifying properties. Rahali et al. (2000) determined the amino acid sequence at an oil/water interface and concluded that the amphiphilic character is more important to the emulsification properties than the peptide's size.

Foaming capacity and stability

Foaming requires the ability to rapidly adsorb from a protein to the interface, thereby lowering the surface tension. Therefore, the adsorption rate, along with the ability to unfold and rearrange at the surface, has been reported as one of the most critical factors for foaming (Alolod et al., 2019). In this study, the highest foam capacity was found in the gelatin from SBS (30.47 ± 0.01%). Gelatin was followed by peptide fractions of BF2 (16.48 ± 0.05%) and BF3 (15.8 ± 0.03%) (Figure 3). There was no foam expansion in the BF1 peptide fraction, and a stable structure could not be obtained. The results were higher compared to the results obtained for the gelatin hydrolysates from Oneknife Unicornfish skin at the same concentration (0.5%) (Alolod et al., 2019) and lower compared to the results obtained for the collagen hydrolysates from the waste mixtures of 10 different fish (Zamorano-Apodaca et al., 2020). As the main protein, gelatin has the highest foaming capacity. As given in this study, foaming depends on molecular weight, and the foaming capacity of the peptide fractions with low molecular weight is also low compared to gelatin. Hence, proteins/peptides with low molecular weight cannot rearrange their structure at the air-water interface; therefore, they cannot exhibit the power required for foaming (Hajfathalian et al., 2018). In terms of foaming stability, the highest value was also obtained for the gelatin from SBS (10.40 ± 0.07%). Gelatin was followed by BF3 (3.23 ± 0.03%) and BF2 (0.64 ± 0.02%). The foam formed by gelatin was more stable compared to the peptide fractions and there was a decrease in foaming stability as the molecular weight decreased (Figure 3).

Similar results were obtained by Zamorano-Apodaca et al. (2020) and Alolod et al. (2019). The observed foaming instability is possibly related to the inability to form strong films due to the peptides with low molecular weight found in peptide

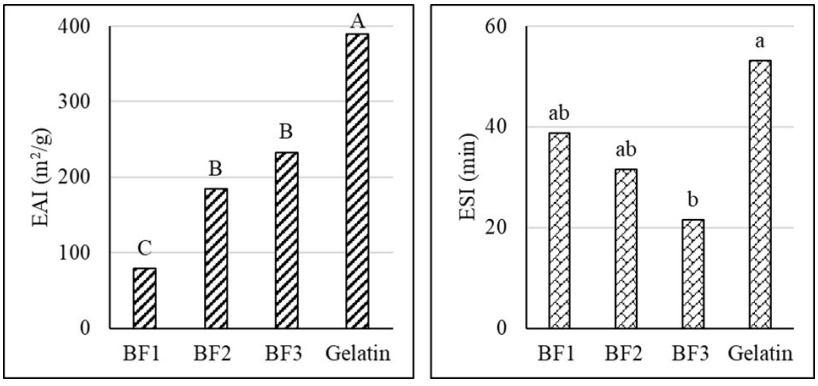

Figure 2. The emulsifying activity index (EAI) and emulsifying stability index (ESI) values of gelatin and peptide fractions. BF1: ≤5 kDa, BF2: 5-10 kDa, BF3: ≥10 kDa. Different letters indicate significant statistical differences between samples ($p < 0.05$).

fractions, low protein-protein interactions, and the inability to stabilize the air cells in the foam (Halim et al., 2016).

Fat binding capacity

Fat binding capacity is an important functional property of proteins in the food production system. It refers to an ability of proteins to absorb fat and hold it against the force of gravity within the protein matrix. The fat binding capacity of gelatin and peptide fractions is displayed in Figure 4. The highest fat binding capacity value was obtained from BF3 (9.39 ± 0.06 mL/g) peptide fractions, while the lowest value was obtained from the gelatin from SBS (2.06 ± 0.04 mL/g).

The fat binding capacity of the BF3 peptide fraction was approximately three times greater compared to gelatin and other peptide fractions. Enzymatic hydrolysis increased the fat binding capacity of peptides compared to gelatin, the main protein (Figure 4). The fat binding capacity of fish protein hydrolysates was reported to be in the range of 1.0-10.8 mL/g (Halim et al., 2016). The values obtained in this study are within the specified range. A low degree of hydrolysis was reported to provide high-fat binding capacity, and the bulk density of proteins and enzyme/substrate specificity affected the fat binding capacity of hydrolysates (Razali et al., 2015). Fat binding capacity of peptides was reported as an essential factor affecting taste of products, especially in meat and confectionery products (Halim et al., 2016).

3.5 Antioxidant properties

2,2-Diphenyl-1-Picryl Hydrazyl (DPPH) activity

DPPH free radical scavenging activities of the gelatin peptide fractions are displayed in Table 1. The highest DPPH activity

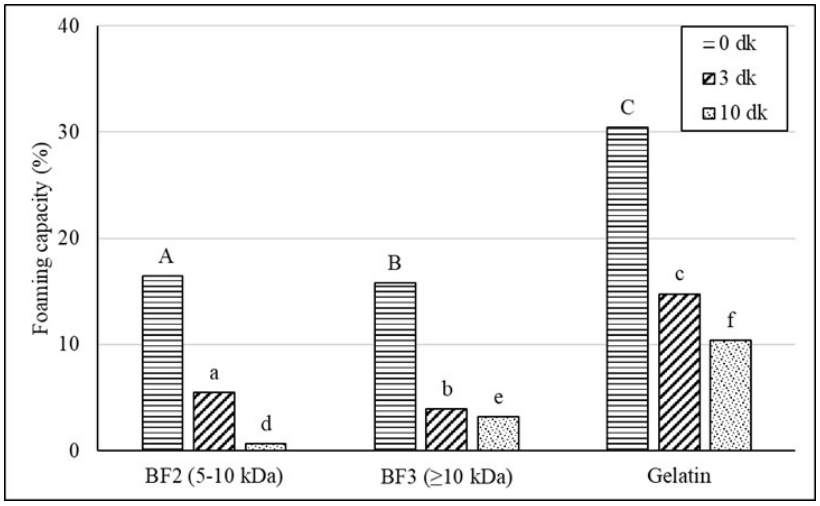

Figure 3. Foaming capacity and stability of gelatin and peptide fractions. Foaming capacity referred to the foaming expansion that occurred at 0 minutes while foaming stability referred to the foaming expansion that occurred at 10 minutes. Different letters indicate significant statistical differences between samples ($p < 0.05$).

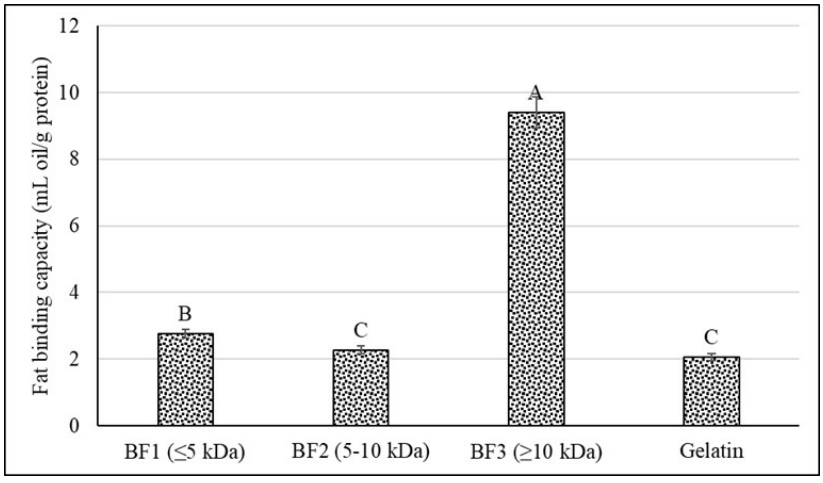

Figure 4. The fat binding capacity of SBS gelatin and peptide fractions. Different letters indicate significant statistical differences between samples ($p < 0.05$).

value belonged to BF1, and the lowest value was obtained for BF3. Peptide fractions with low molecular weights had higher DPPH activities. It was observed that the DPPH activity decreased as the molecular weights of the peptides increased (Table 1). In the study conducted by Zamorano-Apodaca et al. (2020), the DPPH free radical scavenging activity was found as 66% for the peptide fractions between 1-5 kDa. Peptides with low molecular weight could efficiently react with free radicals and inhibit the lipid peroxidation cycle spread. Therefore, they exhibit higher antioxidant activity (Ranathunga et al., 2006). Besides, the high DPPH radical scavenging activity of the peptide fractions has been reported to be usually associated with a high level of hydrophobic amino acid content (Karoud et al., 2019).

Ferric Reducing Antioxidant Power (FRAP)

The ferric reducing antioxidant power analysis of the gelatin peptide fractions is presented in Table 1. The BF1 (≤ 5 kDa) peptide fraction showed higher antioxidant activity than the other peptide fractions. All peptide fractions exhibited ferric reducing power at the hydrolysate ranges of 23.68-42.04 mmol Fe^{+2}/g and 13.15-22.98 mmol trolox/g. Similar results were obtained for the gelatin hydrolysates from Oneknife Unicorfish skin (Alolod et al., 2019) and gelatin hydrolysates from Sole and squid skin (Giménez et al., 2009).

The hydrolysis of the gelatin chains into the peptides may result in the release of sequences with antioxidant properties and the release of previously hidden amino acid residues and side chains with antioxidant activity (Giménez et al., 2009). In this study, the BF1 (≤ 5 kDa) peptide fractions exhibited very high antioxidant properties in both DPPH radical scavenging activity and FRAP, suggesting that they may be antioxidant agents of various products.

3.6 In-vitro cytotoxic activity

Recently, interest in natural anticancer drugs has increased due to the emergence of negative side effects of synthetic cancer drugs and the expensiveness of these drugs. Cytotoxic activity against cancer cells is considered when hydrolysate is added to the medium after the cells have grown for 24 hours. The *in vitro* cytotoxic activity of gelatin peptide fractions is displayed in Figure 5. The highest viability inhibition was detected in the peptide fraction of BF1 (16.58 ± 0.02%) at a concentration of 20 mg/mL. At the same concentration, the inhibition occurred as 10.12 ± 0.03% and 2.56 ± 0.02% in the BF2 and BF3 peptide fractions, respectively.

In general, a higher level of inhibition is achieved as the peptide's size decreased; and the inhibition increased as the concentration level increased in the peptide fractions of the same size (Figure 5). In a similar research, Alemán et al. (2011) investigated the cytotoxic effect of gelatin hydrolysates from squid produced with different enzymes on MCF-7 human breast carcinoma and U87 glioma cell lines and found the highest cytotoxic effect in esperase and alcalase enzymes. In another study (Mirzapour-Kouhdasht et al., 2021) containing hydrolysation of barred mackerel gelatin with alcalase, it was found that 6 mg of fractions < 3 kDa exhibited approximately 5% inhibition on Caco-2 cell line. Moreover, it was reported that the cytotoxic activity was affected by the type of enzyme used in the production of hydrolysate. Similar results were obtained by Mirzapour-Kouhdasht et al. (2020) regarding the peptide fractions belonging to barred mackerel's gelatin (*Scomberomorus commerson*) in terms of their effect on MCF-7 human breast carcinoma. There are studies examining the anticancer effect of seafood-derived protein hydrolysates and peptides on different types of cancer (colon, prostate, breast, etc.), and it is stated in these studies that Pro, Gly, Lys, Arg, and Tyr amino acids are responsible

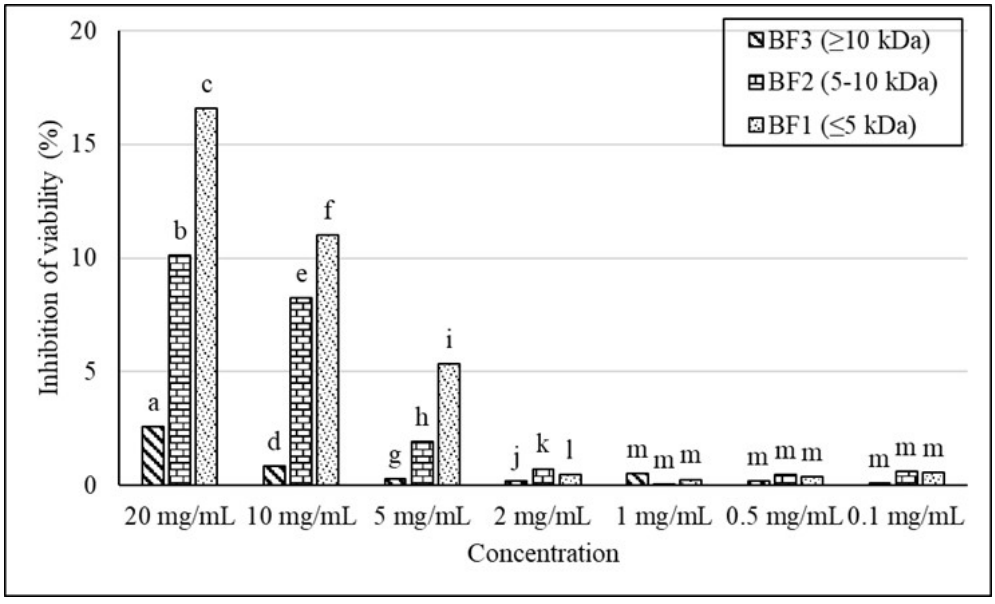

Figure 5. In vitro cytotoxic activity of gelatin peptide fractions on Caco-2 cell line. Different letters indicate significant statistical differences between samples ($p < 0.05$).

for the anticancer activity (Ishak & Sarbon, 2018; Mirzapour-Kouhdasht et al., 2021; Yaghoubzadeh et al., 2020) . Especially low molecular weight peptides interact easily with cancer cell components and increase anticancer activity as they have high mobility and spread (Jumeri & Kim, 2011). Therefore, the use of gelatin hydrolysates and its peptides, which are especially rich in Pro and Gly amino acids, as anticancer agents emerges as an important alternative.

4 Conclusions

In the present study, bioactive (antioxidant and cytotoxic) and functional (emulsion, foaming and fat binding) properties were determined for the peptide fractions obtained from the gelatins from SBS. The results indicate that peptide fractions with low molecular weight have high antioxidant effects and could be used as antioxidant agents in various products. Besides, peptide fractions with low molecular weight also have the potential for anticancer activity. In addition, as the molecular weight of fish gelatin hydrolysates increases, their functional properties show better values. The amount of fish production in the world increases every year; therefore, the amount of waste increases. The fish waste stands out as a raw material to produce bioactive and functional food ingredients. For this purpose, analysis of the conditions regarding different enzymes and processes and optimization studies are essential. There is a need for different and further studies to determine the functional and bioactive properties of the waste deriving from the sea bass.

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Supplementary Material

Supplementary material accompanies this paper.

Figure S1. The degree of hydrolysis for the gelatin hydrolysate from the sea bass skin.

This material is available as part of the online article from https://www.scielo.br/j/cta