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# Collagen extracts from blue cannonball jellyfish (*Stomolophus meleagris*): Antioxidant properties, chemical structure, and proteomic identification

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**ORIGINAL ARTICLE**

## Abstract

The demand for jellyfish is increasing mainly because of its health benefits. This study focuses on extracting and characterizing pepsin-soluble collagen extracts from blue cannonball jellyfish (*Stomolophus meleagris*) mesoglea. Jellyfish mesoglea's amino acids were analyzed, and collagen extracts were obtained using pepsin (24 h, 4°C) and then characterized. Collagen extracts contain high amounts of glycine and hydroxyproline. The extracts obtained in this study exhibited electrophoretic bands corresponding to collagen "a" and "b" chains, and experienced denaturation at 23.9°C and an enthalpy change of 0.078 J/g. The Fourier transform infrared spectra exhibited peaks associated with specific collagen tripeptides, and the proton nuclear magnetic resonance spectra showed the characteristic resonance of collagen-like triple helices. Proteomic studies identified the presence of actin, myosin, and collagen type IV. The collagen extracts exhibited higher *in vitro* ability to trap free radicals, reduce ferric power, and inhibit erythrocyte homolysis than commercial marine fish collagen. The results presented in this paper show the properties of collagen extracts, highlighting their potential application as an antioxidant or a supplement in the food industry.

*Keywords*: antioxidant; collagen; jellyfish; proteins; proteomic

## Introduction

The global rapid growth and reproduction of jellyfish have led to new fisheries and increased the investigation of these organisms as sources of bioactive and functional compounds for novel foods (Bleve *et al.,* 2019). However, owing to the unknown importance of jellyfish biocomponents, until 2020, the Food and Agriculture Organization of the United Nations (FAO, 2024) only reported specific catch statistics for the most caught species of jellyfish (*Rhopilema* spp. and *Stomolophus meleagris*). According to FAO (2024), the total marine capture of jellyfish in 2022 was approximately 248,000 tons.

*S. meleagris,* the cannonball jellyfish, has been a highly profitable fishery and alternative for artisanal fishermen

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worldwide because of decreasing traditional resources and the low cost of capturing this marine organism (Chiarelli *et al.*, 2023). Cannonball jellyfish are distributed mainly in the northwestern Pacific, from California to Ecuador, and in the Gulf of Mexico, representing a significant commercial opportunity for those primarily dependent on fishing (Gómez-Salinas *et al.*, 2021). The approximate number of cannonball jellyfish caught in Mexico between 2018 and 2024 averaged about 46,000 tons (Cruz, 2024).

Adding value to coastal fisheries is a basic factor in developing the fisherman population segment. The high commercial value of jellyfish in Asia is attributed to their therapeutic properties (Brotz *et al.*, 2021; D'Ambra and Merquiol, 2022). Still, it is necessary to find or develop market niches and strategies to improve economies of the fishermen community (Brotz *et al.*, 2021).

Jellyfish are a low-calorie food and a low-cost source of proteins (Chiarelli *et al.*, 2023). The muscle of jellyfish comprises, among other proteins, myosin, paramyosin-like protein, and actin (Tanaka *et al.*, 2018). However, the most significant component of the edible portion of jellyfish is collagen, protein in the connective tissue (Raposo *et al.*, 2022).

Jellyfish gelatin and collagen peptides are evaluated as foods, and it is established that jellyfish collagen could be used as human health supplements (Chiarelli *et al.*, 2023). Moreover, jellyfish collagen (James *et al.*, 2023; Upata *et al.*, 2022) and gelatin (Esparza-Espinoza *et al.*, 2023) have high antioxidant activity, making these organisms a healthy food source of antioxidant compounds. However, information is still scarce on the components responsible for the beneficial properties of cannonball jellyfish proteins, which must be studied extensively to understand their complete potential application.

Jellyfish mesoglea tissue could be solubilized by limited proteolysis with pepsin (Barzideh *et al.*, 2014; James *et al.*, 2023; Kimura *et al.*, 1983; Nagai *et al.*, 1999), which could induce conformational protein changes during precipitation and dissolution, creating a protein complex with a different structure and molecular weight (MW). Proteomic studies can deliver the qualitative and quantitative information necessary to identify proteins, although these analyses require sophisticated equipment and software to interpret their data (Lueyot *et al.*, 2021). To date, no in-depth chemical–structural analysis of pepsin-solubilized collagen extracts from blue cannonball jellyfish has been performed. Proteomic assays using database search algorithms can identify the proteins present, and their potential biological activity can be predicted. These *in silico* assays are the first step in an extensive study of jellyfish proteins, facilitating *in vitro* and *in vivo* assays and reducing their time and costs (Lueyot *et al.*, 2021).

There are few scientific reports regarding *S. meleagris* from Mexican waters. Therefore, this study aimed to examine *S. meleagris* found along the Gulf of California coast for value-added collagen products. The study found that pepsin collagen from this species contains compounds with high antioxidant activity and its efficacy surpasses that of commercial fish marine collagen. This research also presents, for the first time, the results of nano-liquid chromatography/tandem mass spectrometry analyses of pepsin-solubilized collagen proteins from *S. meleagris*. The *in silico* peptide composition of collagen extracted from this Mexican species indicates potential directions for future research and applications.

## Materials and Methods

### Materials

In all, 50 fresh blue cannonball jellyfish (*S. meleagris*) specimens (weight: 500–1,000 g; length: 12–17 cm) were caught off the coast of Bahía Kino, Sonora, Mexico (28°48′ N, 115°57′ W). The specimens were washed to remove insoluble material. The mesoglea tissue was dissected from the skin tissues and cut into small pieces at 4°C, packed in polyethene bags, and stored at -20°C for no more than 30 days. All chemicals employed were of analytical grade and purchased from Baker (Toluca, Mexico City, Mexico), Sigma-Aldrich (St. Lous, MO, USA), and ABATEC (Guadalajara, JAL, Mexico).

### Methods

#### *Amino acid profile*

Amino acid analysis was conducted using reverse-phase high-performance liquid chromatography (RP-HPLC; Model GmbH Hewlett-Packard, Agilent Technologies Inc., Santa Clara, CA, USA) following the specifications reported by Vázquez-Ortiz *et al.* (1995). Freeze-dried samples, 6 mg, were homogenized with 100 mL of performic acid. Performic acid was prepared by mixing 1 mL of 30% hydrogen peroxide with 19 mL of 97% formic acid and then maintained in a closed container at room temperature for 2 h. After preparation, the performic acid was cooled to 0°C and added to the sample. Following this, 0.9 mL of cold water was added after keeping the mixture at 0°C for 2.5 h. An aliquot of this mixture (250 µL) was lyophilized for proline (Pro) and hydroxyproline (Hyp) analysis.

Subsequently, the sample was hydrolyzed under pressure at 110°C for 18 h using 6-M hydrochloric acid and

sodium thioglycolate (in a 1:1 volume ratio). The hydrolysate was neutralized with 4-N sodium hydroxide. An aliquot part was mixed with an equal volume of 10 mg/mL of L-α-amino n-butyric acid, which served as an internal standard. This mixture was vortexed for 1 min with four parts of potassium borate buffer (pH 10.4) and *o*-phthalaldehyde (OPA, in a 1:1 volume ratio). Immediately after the preparation, 20 µL of this solution was injected into a reverse-phase column (C18 octadecyl dimethylsilane, 100 × 4.6 mm) coupled to a pre-column (30 × 4.6 mm) packed with the same material. Amino acids were evaluated by employing a fluorescence detector, and the resulting peaks were evaluated using the ChemStation program. The elution process involved two buffers at a flow rate of 1.0 mL/min for 25 min. Fluorescence was measured at wavelengths of 330 nm (excitation) and 418 nm (emission). Peak areas and retention period were compared to those of a mixture of commercial amino acid standards.

An analysis was performed in triplicate, and the results were expressed as the number of amino acid residues per 1,000 residues.

*Extraction of mesoglea collagen*

Mesoglea collagen extraction was performed as described in previous studies (James *et al.*, 2023; Nagai *et al.*, 1999) with modifications. All processes were achieved at 4°C. Small pieces of jellyfish mesoglea were suspended in NaOH 0.1-M 1:5 (w/v) solution. The samples were stirred for 24 h and centrifuged at 9,000×*g* for 30 min. The pellets were rinsed until the pH dropped to 7 and freeze-dried. Afterward, the samples were sequentially treated with acetic acid 0.5 M (1:5 w/v) and pepsin (10 mg/g sample in 0.5 acetic acid; 1:5 w/v). At each step, after stirring for 24 h, the samples were centrifuged (6,000×*g* for 15 min). The protein solution was then dialyzed against water using a cellulose membrane with a 50-kDa molecular weight cut-off. The collected sample was freeze-dried.

The sample's collagen concentration was determined by its hydroxyproline by the method adhered to the procedure established by Vázquez-Ortiz *et al.* (2004) and protein content according to the specification established in the Association of Official Analytical Chemists (AOAC, 2000). The proline and hydroxyproline content were quantified using a Hewlett-Packard HPLC system (model GMBH, Waldbronn, Germany) equipped with a fluorescence detector. The pre-column derivatization method abided the procedure established by Vázquez-Ortiz *et al.* (2004). In brief, a 0.4-M borate buffer at pH 10.4 was combined with a 250-µL aliquot of lyophilized sample to achieve a total volume of 1 mL. From this solution, 250 µL was mixed with 250 µL of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole solution (2.0 mg/mL in methanol). The resulting mixture was then heated at 60°C, and precisely 5.0 min later, 50 µL of 0.1-M HCl was added. This mixture was cooled to 0°C for 30 min, after which 10 µL of the sample was manually injected into HPLC system (Varian, Inc., Palo Alto, CA). Amino acid separation was conducted under the same conditions as described previously.

Crude protein was quantified using the Dumas method in accordance with the official AOAC method (993.13), employing a LECO FP-2000 nitrogen/protein analyzer (St. Joseph, MI, USA). This method involves high-temperature combustion to release nitrogen from the sample, which is subsequently measured through thermal conductivity detection. A conversion factor of 5.8 was applied to convert nitrogen (N) content into a percentage (%) of protein in the sample (Doyle *et al.*, 2007).

*Molecular weight*

The molecular mass of collagen extracts from freeze-dried jellyfish mesoglea were analyzed with sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) using 7% acrylamide separating gel. Protein extract samples and standard collagen type I from calf skin (Sigma-Aldrich) were dissolved in 0.02-M sodium phosphate, pH 7.2, containing 1% SDS and 3.5-M urea (Kimura *et al.*, 1983). Proteins (20 µL, 12 µg) were loaded onto electrophoresis gel, which was developed in Miniprotean® IIIBio-Rad electrophoretic equipment (Hercules, CA, USA) at 120 V for 90 min. Molecular weight was determined using a protein molecular weight marker kit (Sigma-Aldrich). The gels were analyzed using a densitometer (Bio-Rad GS-700, Hercules).

*Thermal analysis*

The thermal analyses of jellyfish mesoglea collagen extracts and calf skin collagen were conducted with differential scanning calorimetry (DSC) using a Perkin Elmer model 8500 DSC system (Perkin Elmer, Waltham, MA, USA). An approximately 6-mg freeze-dried sample was placed in an aluminum pan at a heating rate of 10°C/min from 10°C to 120°C under a nitrogen atmosphere. An empty capsule was used as a reference. The equipment's software automatically calculated the denaturation temperature (Td) and enthalpy (ΔH) of the jellyfish mesoglea protein extracts. The enthalpy was expressed as joules per g of sample. Analyses were performed in triplicate.

*Spectroscopic methods*

The Fourier transform infrared (FTIR) spectrum of jellyfish mesoglea collagen extracts (0.2 mg in 10-mg potassium bromide) was obtained with a Perkin–Elmer FTIR spectrometer (Frontier MIR/FIR, Waltham, MA, USA), with an average of 16 scans over a spectral range of 4,000–400 $cm^{-1}$, at a resolution of 4 $cm^{-1}$ (Esparza-Espinoza *et al.*, 2023).

The proton nuclear magnetic resonance ($^1$H-NMR) spectra of jellyfish mesoglea collagen extracts were obtained using a Bruker Avance 400 nuclear magnetic spectrometer (Billerica, MA) at 400 MHz and 25°C. Freeze-dried samples (1 mg) were dissolved in 0.5 mL of deuterated water ($D_2O$ at 99.6% 2H atom) and 0.5 mL of deuterated potassium hydroxide solution (KOD 40% in $D_2O$). Dimethylsilapentane-5-sulphonic acid (DSS) was used as a reference, with a 20-ppm spectral window (Esparza-Espinoza *et al.*, 2023).

Three spectra were analyzed for each spectroscopic method to acquire reliable, characteristic and valid results.

*Nano-liquid chromatography/tandem mass spectrometry (nano-LC-MS/MS) analysis*

Jellyfish mesoglea collagen extracts were dissolved and identified with a nano-LC-MS/MS platform (Ultimate 3000 nano-UHPLC system and Orbitrap Q Exactive HF mass spectrometer with Nanospray Flex ion source; Thermo Scientific, Waltham, MA, USA), as reported previously (Esparza-Espinoza *et al.*, 2023). Briefly, the pepsin protein extracts were poured into a column (C18 SPE, 100Å, 100 × 2 cm, 5 mm; Thermo Scientific) with 0.1% formic acid to remove salt, and 1 mg of the sample was loaded into Nanoflow UPLC with a flow rate of 250 nL/min. The MS/MS scan (300–1,650 m/z, resolution 60,000 at 200 m/z, 3e6) was operated in the top 20 mode using the following settings: resolution 15,000 at 200 m/z, automatic gain control target 1e5, maximum injection time of 19 ms, normalized collision energy at 28%, and an isolation window of 1.4 Th. The charge state exclusion parameters were set to unassigned, 1, >6, and a dynamic exclusion of 30 s. Raw MS files were analyzed and searched against the jellyfish protein database based on the species of the samples using PEAKS Studio 8.5. The parameters were set as follows: the protein modifications were carbamidomethylation (CAM; fixed) and methionine oxidation (variable), and the enzyme specificity was pepsin. There were two maximum missed cleavages; the precursor and ion mass tolerance were 10 ppm and 0.5 Da, respectively.

*Determination of in vitro antioxidant activity*

The antioxidant activity of jellyfish mesoglea collagen extracts and commercial fish marine collagen (0.75 mg/mL) was evaluated by chemical and cellular assays. All measurements were performed in triplicate.

The sample's 2,2'-azino-bis (3-ethylbenzothiazolin-6-sulphonic acid) (ABTS●+) radical scavenging activity was measured (Re *et al.*, 1999). The working solution was prepared by mixing 7.4-mmol ABTS and 2.45-mmol potassium persulphate. After the ABTS●+ was produced in a dark room at 25°C for 6 h and diluted with methanol, pepsin protein extracts were added. Then, the absorbance was taken at 734 nm using a 96-well microplate reader (Thermo Fisher Scientific Inc., Multiskan GO, New York, NY, USA).

The ferric reducing antioxidant power (FRAP) assay was performed according to Benzie and Strain's (1996) method, adapted to microplate equipment. The working solutions consisted of a 300-mM acetic acid–sodium acetate buffer (pH 3.6), 20-mM $FeCl_3 \cdot 6H_2O$, and 10-mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) in 40-mM HCl (10:1:1 ratio). An aliquot of 20 mL of the samples was mixed with 280 mL of FRAP working solution and incubated at 25°C in the dark for 30 min before its absorbance was recorded at 638 nm using a microplate reader.

The ability of jellyfish collagen extracts to quench the oxygen radicals generated by 2,2'-azobos(2-amidinopropane) (AAPH) was measured with the oxygen radical absorbance capacity (ORAC) technique (Prior *et al.*, 2003). The inhibition of fluorescence decay after mixing collagen extracts (100 mL) with 75-mM phosphate buffer, pH 7.3 (1.7 mL), 0.36-M AAPH (0.1 mL), and 0.048-mM fluorescein (100 mL) was evaluated for 60 min at 37°C at 485 nm (excitation) and 520 nm (emission) using a Cary Eclipse spectrophotometer (Agilent Technologies, Mexico City, Mexico).

The results for ABTS, FRAP, and ORAC were expressed as micromoles of Trolox equivalent per gram of the sample (mmol TE/g). A standard curve was prepared using different Trolox concentrations.

Jellyfish pepsin collagen extracts' anti-hemolytic capacity against AAPH was evaluated as described by Lu *et al.* (2010). Briefly, a pure solution of erythrocytes was resuspended in 10-mM phosphate-buffered saline (PBS), pH 7.4 (ratio 5:95, v/v). Three different mixtures were prepared: (1) resuspended erythrocytes (100 mL) + protein extracts (100 mL) + AAPH (100 mL); (2) resuspended erythrocytes (100 mL) + AAPH (100 mL) + 100-mL PBS; and (3) resuspended erythrocytes (100 mL) + 200-mL PBS. After the mixtures were incubated in a dark room at 37°C for 3 h, PBS (1 mL) was added to the solution and centrifuged (1,500×*g*, 10 min, 4°C). In all, 300 µL of supernatant was transferred to 96-well microplates, and absorbance was read at 540 nm using a spectrophotometric microplate reader. The intact erythrocytes were established, and the results were expressed as percentage inhibition of hemolysis.

### *Statistical analysis*

The experimental design of this work was intended to reduce variation among replicates. All experiments were

performed in triplicate. The results from the amino acid profile, antioxidant assay, and thermal analysis data are presented as mean values ± standard deviations and were analyzed using one-way analysis of variance (ANOVA). Significant differences ($p < 0.05$) between samples were evaluated using Student's *t*-test. Analysis was performed using XLSTAT 2024.3. Data from electrophoretic analysis, FTIR, and $^1$H-NMR and proteomics were evaluated by descriptive statistics.

## Results and Discussion

### Amino acid profile of jellyfish mesoglea

Fresh jellyfish mesoglea contained about 75.7±0.7% protein based on ash-free dry weight. Their amino acid composition expressed as residues/1,000 residues (Table 1) revealed high concentrations of glycine (301±1.4), proline (84±1.1), and hydroxyproline (38±0.3). *S. meleagris* mesoglea showed almost 1.7 times higher content of hydroxyproline content (3.8%) than those reported for other jellyfish species, such as *Cassiopea andromeda* (1.6%) and *Catostylus tagi* (1.7–2.2%) (Guttuso *et al.*, 2025). Moreover, considering that hydroxyproline is the best means for collagen estimation, it was found that the percentage of collagen detected in this work in the jellyfish mesoglea (35.4%) was comparable to estimated collagen from edible *Rhizostoma hispidum* jellyfish body (about 38%) (Khong *et al.*, 2016).

### Properties of the mesoglea's pepsin collagen extracts

*Electrophoretic pattern*

The electrophoretic pattern of *S. meleagris* jellyfish pepsin collagen extracts, under the electrophoretic conditions employed, revealed two "a" chain-like components with a very similar migration and a molecular mass of about 117 kDa (Figure 1, lane B). The migration of jellyfish pepsin collagen extracts was like that of calf skin collagen (Figure 1, lane C), specifically type I a1, but not a2, which may be due to variations in amino acid composition. Similar findings were reported for *Chrysaora spp.* (Barzideh *et al.,* 2014). This behavior could be attributed to the possibility that both chains originated from distinct molecular collagen types (Kimura *et al.*, 1983). It is observed that some jellyfish collagens are comparable to collagen type I (Rastian *et al.*, 2018). In contrast, others align more closely with type II (Barzideh *et al.*, 2014), and some are related to types IV and V (Lueyot *et al.*, 2021).

Furthermore, a high molecular weight band was detected at around 199 kDa in *S. meleagris* and above 200 kDa in calf skin, which was associated with the b collagen component. It is established that "α" chains build trimeric

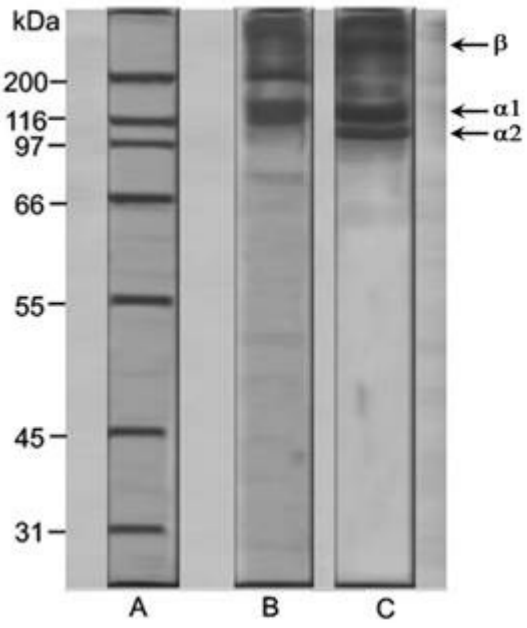


**Figure 1. Electrophoresis profile of collagen extracts from cannonball jellyfish collagen. Lane A: molecular weight marker. Lane B: jellyfish pepsin collagen extracts. Lane C: calf skin collagen.**

molecules, which coil together into a triple-helical conformation. In contrast, "β" chains reveal the presence of an oligomer with a higher degree of order (Eyre *et al.,* 2019). Then, the differences in "α" and "β" chains imply that the analyzed collagen possessed a different degree of cross-linking. Additionally, the presence of faint bands may arise from residual non-collagen proteins, a finding further validated by proteomic analysis, as discussed below.

*Amino acid profile*

The amino acid profile of jellyfish pepsin collagen extracts was assessed using HPLC in conjunction with amino acid standards (Figures 2A and 2C). The use of OPA for the separation of amino acids facilitated the identification and quantification of 15 different amino acids (Figure 2B). The chromatograms for proline and hydroxyproline within the jellyfish collagen extract are presented in Figure 2D.

The individual content of free amino acids in jellyfish pepsin collagen extracts and the data expressed as residues/1,000 residues, presented in Table 1, are the average values from a triplicate analysis. Glycine (Gly) was the major amino acid with 335 residues per 1,000. Other high-content amino acids were glutamic acid (Glu, 99 residues per 1,000), alanine (Ala, 83 residues per 1,000), proline (Pro, 8 residues per 1,000), and aspartic acid (Asp, 76 residues per 1,000). Hydroxyproline was also detected in

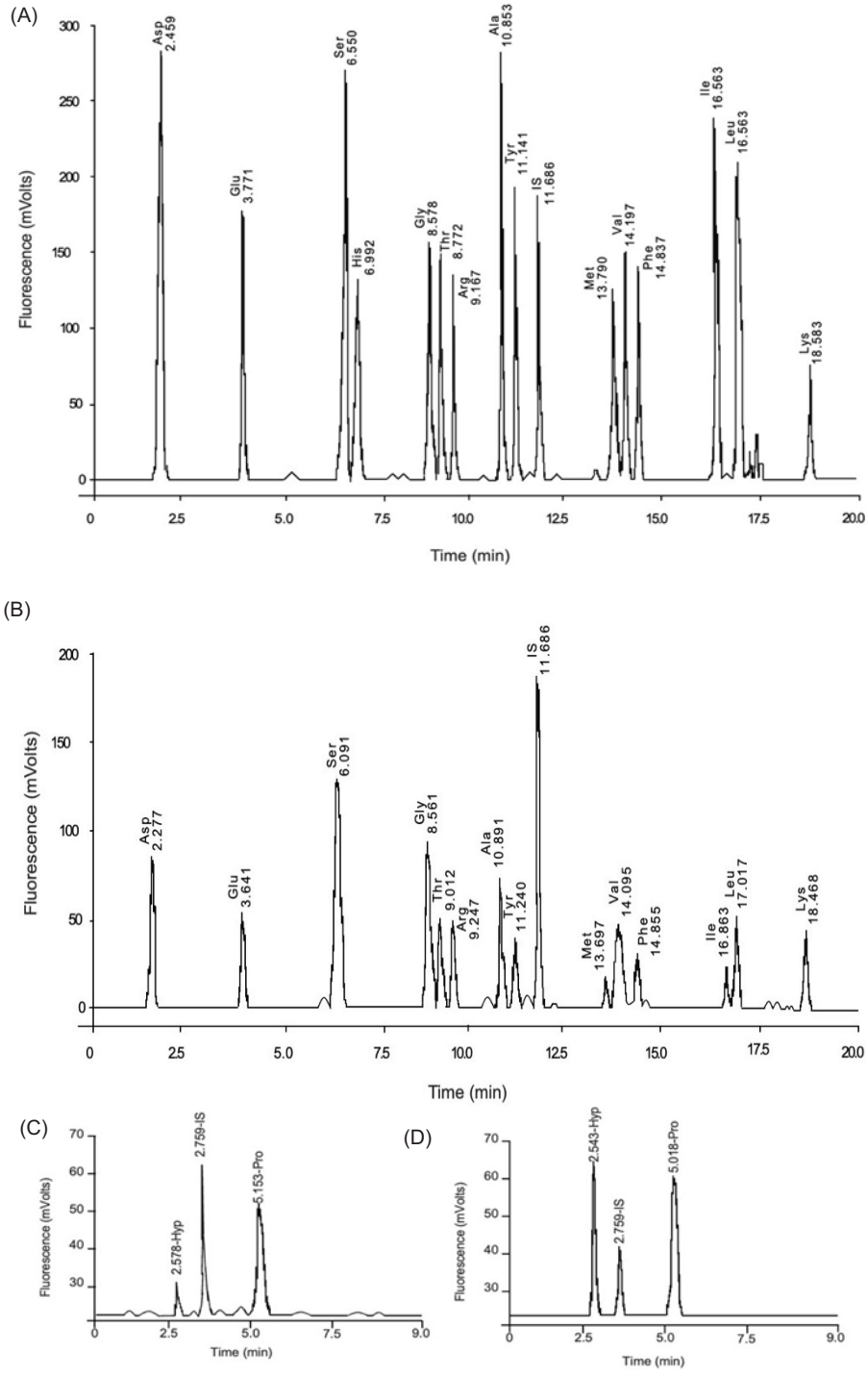


**Figure 2. Electrophoresis profile of collagen extracts from cannonball jellyfish collagen. Lane (A) molecular weight marker. Lane (B) jellyfish pepsin collagen extracts. Lane (C) calf skin collagen.**

considerable amount (49 residues per 1,000). These data indicate that collagen is the major protein in pepsin collagen extracts. Like *Chrysaora* spp. (Barzideh *et al.*, 2014) and *Rhopilema esculentum* (Sudirman *et al.*, 2023), histidine was not found (Table 1).

From the hydroxyproline content of collagen extracts, it was estimated that the collagen content was approximately 45.6%. Similar values were reported for the same jellyfish species (46.4%) (Nagai *et al.*, 1999) as well as *Rhizostoma pulmo* (47%) (James *et al.*, 2023), but

**Table 1. Amino acid composition (number of residues/1,000 amino acids) of the cannonball jellyfish (*Stomolophus meleagris*) whole tissue and pepsin-soluble collagen extracts, compared to calf skin collagen type I and different jellyfish collagen extracted from species reported in the literature.**

| Amino acid | *Stomolophus meleagrisa* | | *Calf skin collagen*[a] | *Stomolophus meleagris umbrella*[b,c] | *Chrysaora sp. Umbrella*[b,d] | *Rhopilema esculentum*[e] |
|---|---|---|---|---|---|---|
| | Whole tissue | Collagen[b] | | | | |
| Gly | 301 (1.4) | 335 (1.2) | 329 (1.9) | 309 | 320 | 325 |
| Lys | 39 (0.8) | 38 (0.6) | 34 (0.4) | 38 | 17 | 30 |
| His | ND | ND | 3 (0.1) | 2 | ND | ND |
| Arg | 53 (0.8) | 55 (0.6) | 50 (0.7) | 52 | 58 | 56 |
| Asp | 78 (1.1) | 76 (1.3) | 41 (0.6) | 79 | 76 | 77 |
| Glu | 110 (1.2) | 99 (1.1) | 73 (0.9) | 98 | 101 | 103 |
| Ser | 42 (0.4) | 46 (0.9) | 39 (0.5) | 45 | 44 | 30 |
| Thr | 42 (0.4) | 33 (0.4) | 18 (0.3) | 35 | 34 | 27 |
| Hyp | 38 (0.3) | 49 (0.8) | 95 (1.1) | 40 | 70 | 47 |
| Tyr | 8 (0.2) | 5 (0.3) | 3 (0.1) | 6 | 10 | 7 |
| Ala | 92 (1.1) | 83 (1.3) | 118 (1.2) | 82 | 87 | 101 |
| Val | 34 (0.4) | 28 (0.4) | 21 (0.3) | 35 | 22 | 22 |
| Leu | 36 (0.4) | 34 (0.2) | 28 (0.5) | 34 | 31 | 31 |
| Ile | 21 (0.3) | 19 (0.3) | 15 (0.2) | 22 | 23 | 12 |
| Pro | 84 (1.1) | 81 (1.2) | 119 (1.6) | 82 | 79 | 96 |
| Phe | 13 (0.4) | 9 (0.4) | 7 (0.2) | 10 | 14 | 15 |
| Met | 10 (0.2) | 9 (0.3) | 6 (0.1) | 4 | 16 | 9 |
| Imino acid (Pro + Hyp) | 120 (1.1) | 128 (1.7) | 214 (1.2) | 122 | 149 | 143 |
| Pro hydroxylation (%) | 31 (0.6) | 37 (0.3) | 44 (0.3) | – | – | – |

Notes: ND = not detected.
[a]Values are the mean of triplicate (n = 3). Standard deviations are indicated in parenthesis.
Different letters in imino acid (Pro + HyP) and pro-hydroxylation indicate significant differences ($p < 0.05$).
[b]Pepsin solubilized collagen.
[c]Nagai *et al.*, 1999.
[d]Barzideh *et al.*, 2014.
[e]Sudirman *et al.*, 2023.

*Chrysaora* spp. was reported to have 2.4 times less collagen (19%) (Barzideh *et al.*, 2014).

The amino acid composition of jellyfish pepsin collagen extracts was compared to that of calf skin collagen type I as well as the values reported by other authors (Table 1). *S. meleagris* collagen extracts were determined to have lower Pro and Hyp ($p < 0.05$) than calf skin type I collagen. Moreover, *S. meleagris* collagen extracts showed 1.1 times higher Gly than that of *S. meleagris* exumbrella (Nagai *et al.*, 1999), *Chrysaora* spp. umbrella (Barzideh *et al.*, 2014), and *R. esculentum* (Sudirman *et al.*, 2023), but 1.2 times low values of imino acids than *Chrysaora* spp. umbrella (Barzideh *et al.*, 2014) and *R. esculentum* (Sudirman *et al.*, 2023). Low contents of Pro and Hyp imply that the secondary structure of collagen is represented more by a b-turn/b-shift structure than triple helices (Derkach *et al.*, 2019). The large number of a b-structure can affect the functional properties of the molecule (Karim and Bhat, 2009) as well as its thermal behaviors (Barzideh *et al.*, 2014), as discussed below.

The differences detected among jellyfish's amino acid profile and collagen content may be attributed to specific living conditions (Cadar *et al.*, 2023) and the fishing region's marine environment (Haard *et al.*, 1982).

### *Differential scanning calorimetry*

Differential scanning calorimetry determined the thermal stability of jellyfish pepsin collagen extracts and calf skin collagen. The denaturation temperature (Td) obtained from the heating scan of jellyfish pepsin collagen extracts was 25.1±1.4°C, which was 1.7 times lower ($p < 0.05$) than that of skin calf type I collagen (41.8±1.3°C). Comparing the data with those reported by other authors, Td values were shown to be similar to collagen extracted from *S. nomurai* mesoglea (27°C) (Kimura *et al.,* 1983) and

*S. meleagris* exumbrella (26°C) (Nagai *et al.,* 1999) but 1.5 times lower than that of *Chrysaora* spp. collagen (37.38°C) (Barzideh *et al.,* 2014) and 1.7 times for *Aurelia aurita* collagen (43.7°C) (Balikci *et al.,* 2024). In addition, jellyfish pepsin collagen extracts exhibited a degree of hydrolysis (DH) of 0.078 J/g, which was 67 times lower ($p < 0.05$) than that of calf skin collagen (5.26 J/g). Moreover, the DH values for *S. meleagris* were 23 times lower than *Aurelia aurita* collagen's (1.806 J/g) (Balikci *et al.,* 2024) and 30 times lower than *Chrysaora* spp. collagen (2.35 J/g) (Barzideh *et al.*, 2014). As mentioned, *S. meleagris* jellyfish pepsin collagen extracts had lower imino acid content than the collagen from calf skin ($p < 0.05$) and *Chrysaora* spp. umbrella (Barzideh *et al.,* 2014). The lower DH value of *S. meleagris* mesoglea pepsin collagen extracts could be related to the low stability of the molecule, which could be due to the hydroxyproline and proline content of its structure (Rodríguez *et al.,* 2017) as well as the method of extraction, water content, and the source organism's environmental habitat (Safandowska and Pietrucha, 2013).

### *Spectroscopic analysis*

The jellyfish pepsin collagen extracts' FTIR spectrum (Figure 3) was comparable to that of other jellyfish species (Table 2). Jellyfish pepsin collagen extracts' FTIR spectrum displayed five major peaks of collagen characteristics. The peak associated with the N–H stretching frequency (amide A) was observed around 3,290 $cm^{-1}$. The N–H stretching vibration usually occurs at 3,440–3,400 $cm^{-1}$, and it is reported that when the band position appears at a lower frequency, it suggests that N–H groups are involved in hydrogen bond formation, which can help stabilize the collagen triplehelix (Doyle *et al.*, 1975). The band observed at 2,920 $cm^{-1}$ was related to the asymmetric stretch of $CH_2$ and $NH_3^+$(amide B) (Zhang *et al.*, 2014). Amide I, II, and III waves associated with collagen structure (Riaz *et al.*, 2018) were observed from 1,660 $cm^{-1}$ to 1,200 $cm^{-1}$. CO stretching (amide I) was detected at 1,660 $cm^{-1}$ whereas N–H and C–N torsional vibrations (amide II) were observed at 1,585 $cm^{-1}$. The absorption band around 1,220 $cm^{-1}$ (amide III) is linked to residual CH groups. The triple bands observed around 1,350–1,200 $cm^{-1}$ were caused by specific collagen fingerprint tripeptides (Gly-Pro-Hyp) (Riaz *et al.*, 2018).

The spectra of collagen type I from calf skin showed that peaks of amide I and II had a higher frequency (Table 2). The shift of these peaks to lower frequency observed in jellyfish collagen extracts implied that the molecules had a lower degree of molecular order (Payne and Veis, 1988). Moreover, the index absorption ratio of amide III band at 1,450 $cm^{-1}$ (amide III/A 1,450), associated with a helical structure and detected in the jellyfish collagen extracts, was lower (0.97) than the ratio from skin calf collagen (1.13). These values implied that fewer intermolecular

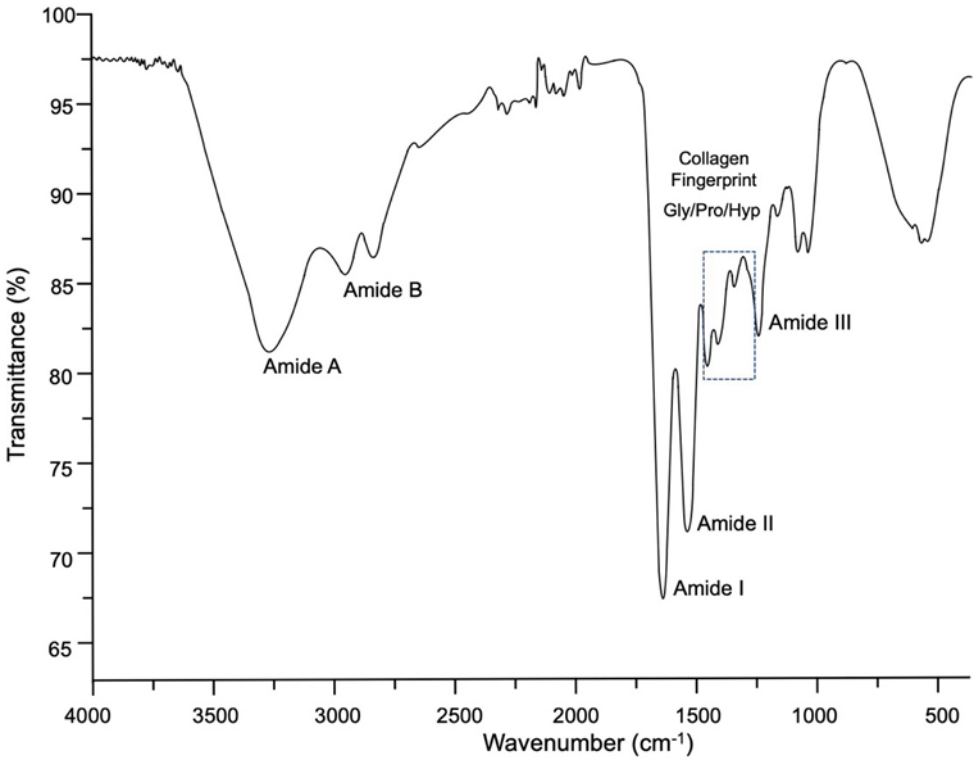


**Figure 3. FTIR spectra of pepsin-soluble collagen extracts from the cannonball jellyfish (*Stomolophus meleagris*).**

**Table 2. Comparison of pepsin-soluble collagen extracts to FTIR band locations ($cm^{-1}$) of the cannonball jellyfish (*Stomolophus meleagris*) and different jellyfish species reported in the literature.**

| Band | *Stomolophus meleagris* | Calf skin collagen | *Aurelia aurita*[a] | *Chrysaora spp.*[b] | *Rhopilema esulentum*[c] |
|---|---|---|---|---|---|
| Amide A | 3,260 | 3,302 | 3,314 | 3,314 | 3,322.1 |
| Amide B | 2,920 | 2,928 | 2,924 | 2,924 | 2,928 |
| Amide I | 1,660 | 1,674 | 1,653 | 1,653 | 1,660.5 |
| Amide II | 1,535 | 1,549 | 1,551 | 1,551 | 1,552.7 |
| Amide III | 1,240 | 1,283 | 1,239 | 1,239 | 1,237.8 |

Notes: [a]Balikci *et al.*, 2024.
[b]Barzideh *et al.*, 2014.
[c]Felician *et al.*, 2019.

cross-links were observed in jellyfish collagen extracts than that in calf skin collagen (Chen *et al.*, 2016). Fourier transfom infrarred (FTIR) correlated positively with Td and DH data.

A comparison of *S. meleagris* collagen's FTIR amide bands to those reported previously for other jellyfish collagen (Table 2) indicated that most bands obtained in our study were similar to those reported from different jellyfish species, with only one exception, amide A, which was detected at a lower wave number. The lower wave number of the amide A band, which is very sensitive to hydrogen bond strength, implies fewer hydrogen bonds from -NH groups (Balikci *et al.*, 2024). This value indicates that the -NH groups of *S. meleagris* mesoglea collagen are involved with other groups through hydrogen bonds to stabilize the collagen's helical structure (Duan *et al.*, 2009; Wang *et al.*, 2007).

The $^{1}$H-NMR spectroscopy is used to detect collagen's triple helix structure (Baum and Brodsky, 1997). The $^{1}$H-NMR spectrum of *S. meleagris*'s collagen showed the characteristic resonance of a collagen-like triple helix (Figure 4). The chemical shift at 3.58 ppm indicated the presence of GlyCαH (Acevedo-Jake *et al.*, 2015) whereas the chemical displacements at 3.55 ppm and 3.10 implied the presence of proline protons bound (ProCdH1,h) (Ovando-Roblero *et al.*, 2023). Meanwhile, the resonance at 2.08 ppm was attributed to hydroxyproline protons (HypCbH1) (Ovando-Roblero *et al.*, 2023).

The intense band at 4–6 ppm indicated the presence of water. Collagen fibers contain ≤0.5-g water/g collagen; this water represents the "bound" water fraction, which interacts with collagen's surface (Fullerton *et al.*, 2006). Chemical displacements at 7.6–7.1 ppm indicated the presence of aromatic protons of pyrrolidine (Sell and Monnier, 1989).

The above observations were consistent with the profiles of amino acid content discussed earlier. The analysis of NMR spectrum, combined with insights from FTIR spectrum, provided strong evidence for the presence of functional groups associated with collagen in extracts. It confirmed the presence of components related to collagen, thereby enhancing our understanding of the structural properties and potential biological significance of the extracted material.

### *Nano-LC-MS/MS analysis*

The proteins determined by nano-LC-MS/MS from *S. meleagris* collagen extracts, summarized in Table 3, are from 3,086 identified spectra (peptide spectrum matches [PSM]), with 707 different peptides belonging to 10 proteins, one of which is collagen type IV. The bioinformatic analysis showed that the collagen detected in this study contained 15 unique peptides. Through LC-MS/MS, the proteomic analysis of gelatin from dried minced jellyfish (*Lobonema smithii*) showed 12 proteins, in which several types of collagen were detected, including collagen type IV alpha 4 chain, collagen alpha 2 (IX chain), collagen type V alpha 2 chain, collagen 1 alpha 2 chain, and collagen alpha-2 (IX chain) (Lueyot *et al.*, 2021).

Jellyfish collagen has a great degree of chemical simplicity, leading to multifaceted and adaptable tissues. Consequently, it has been categorized as collagen type 0 (Faruqui *et al.*, 2023). It is similar in chemical composition to various collagen types (Faruqui *et al.*, 2023) and shares many functions with more specialized collagens (Bowen *et al.*, 2022), such as bioactive (antioxidant or antimicrobial) or functional properties (such as those exhibited by emulsifiers, foaming substances, colloid stabilizers, and biodegradable film-forming materials) (Chiarelli *et al.*, 2023).

## Antioxidant activity

This study applied different assays—ABTS, FRAP, ORAC, and inhibition of erythrocyte hemolysis (antihemolysis)—to examine the pepsin collagen extracts of *S. meleagris* and commercial fish marine collagen

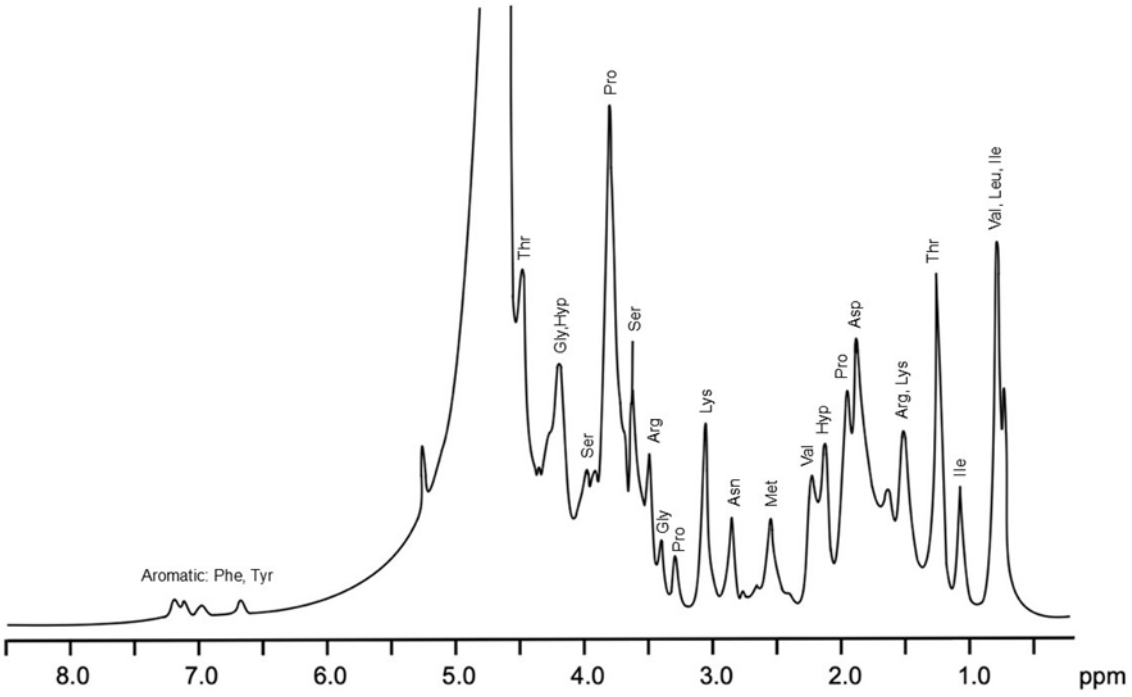


**Figure 4.** **$^{1}$H-NMR spectra of pepsin-soluble collagen extracts from the cannonball jellyfish (*Stomolophus meleagris*).**

**Table 3.** **Proteins identified in *Stomolophus meleagris* jellyfish by nano-LC-MS/MS and bioinformatic analysis.**

| Protein description | Accession No.[a] | Avg. Mass (Da)[b] | PSM[c] | Cov (%)[d] | Score[e] |
|---|---|---|---|---|---|
| Non-muscle actin (*Malo kingi)* | tr\|D1FP00_9CNID | 41,839 | 1,405 | 84 | 651.66 |
| Actin (*Aurelia* spp.) | tr\|A0A2Z5WH75 | 41,834 | 1,403 | 85 | 650.37 |
| Tubulin (*Aurelia aurita*) | tr\|Q7YZL5 | 42,659 | 55 | 49 | 332.29 |
| Histone (*A. aurita*) | tr\|Q5VJP9 | 12,294 | 33 | 69 | 202.11 |
| Myosin heavy chain (*Aurelia* spp.) | tr\|A0A2Z5WH73 | 2,25,197 | 88 | 26 | 171.77 |
| Elongation factor 1-alpha (*A. aurita*) | tr\|R9S2M2 | 51,855 | 21 | 32 | 118.49 |
| Cytochrome C oxidase (*Cassiopea frondosa*) | tr\|Q6VYK4 | 21,787 | 23 | 60 | 65.97 |
| NADH ubiquinone reductase (H+-translocating) (*Chrysaora quinquecirrha)* | tr\|G8DM08 | 21,809 | 15 | 20 | 46.88 |
| DNA polymerase (*Carybdea alata*) | tr\|A0A1B1SJR3 | 28,272 | 4 | 18 | 46.43 |
| Collagen type IV (*Craspedacusta sowerbii*) | tr\|V9GWB0 | 1,28,153 | 17 | 14 | 38.32 |

Notes: Da: Daltons.
[a]Accession number according to the PEAK STUDIO 8.5 database.
[b]Theoretical mass of identified proteins retrieved from the database.
[c]Peptide spectrum matches.
[d]Protein sequence coverage.
[e]Score reported after database search; score > 32 indicates extensive homology at $p < 0.01$.

antioxidant capacity. The results showed that jellyfish pepsin collagen extracts and commercial fish marine collagen could donate hydrogen atoms and electrons (ABTS), reduce $Fe^{3+}$ to $Fe^{2+}$ (FRAP), inhibit oxygen-derived radicals (ORAC), and inhibit free radical-induced hemolysis (anti-hemolysis) (Table 4). According to the comparison of samples, the commercial product was much less active, as shown in Table 4. *S. meleagris* demonstrated 1.3–2.6 times more antioxidant activity than commercial collagen ($p < 0.05$).

Data from jellyfish collagen extracts were comparable to the ABTS values of *Rhizostoma pulmo* jellyfish collagen hydrolysates (2,453 μmol TE/g) (De Domenico *et al.*, 2019) and ORAC data of blue mussel (*Mytilus edulis*) collagen hydrolysates (256–311 μmol TE/g) (Neves

**Table 4. Antioxidant activity of pepsin-soluble collagen extracts of cannonball jellyfish (*Stomolophus meleagris*) and commercial fish marine collagen.**

| Determination | *Stomolophus meleagris* | Commercial fish |
|---|---|---|
| ABTS (µmol TE/g) | 2,916.7 (0.69)[a] | 1,103.4 (0.35)[b] |
| FRAP (µmol TE/g) | 987.1 (15.5)[a] | 548.1 (9.6)[b] |
| ORAC (234.7±28.2 µmol TE/g) | 234.7 (28.2)[a] | 178.3 (16.7)[b] |
| Anti-hemolysis (%) | 46.3 (0.61)[a] | 27.2 (0.43)[b] |

Notes: The values are mean values from triplicate analysis (n = 3). Standard deviations are indicated in parenthesis.
Different superscript letters in the same row indicate significant differences ($p < 0.05$).
TE: Trolox equivalent.

*et al.*, 2022), but the FRAP results were 1.5–4.1 times higher than that for the protein hydrolysates of *L. smithii* jellyfish (240–650 µmol TE/g) (Upata *et al.*, 2022) and the anti-hemolytic capacity was 1.3 times greater than protein extract hydrolysates from *Arthrospira (Spirulina) platensis* (35%) (Zeng *et al.*, 2018).

The studied sample's capacity for inhibiting the activity of unpaired electrons is associated with phenylalanine (Jiang *et al.*, 2018). Meanwhile, its ability to stabilize peroxyl radicals could be due to hydrogen donated by amino acids, such as leucine (Byun *et al.*, 2009). Finally, the presence of glycine and tyrosine in the extracts (Zheng *et al.*, 2016) could protect erythrocytes against AAPH-induced hemolysis. However, more investigations are required to corroborate this.

Bioactive peptides are inactive when they are part of a protein and develop their activity when released by enzymatic action. The bioactive peptides encrypted in the proteome of *S. meleagris* mesoglea's collagen extracts were predicted in silico using the PeptideRanker software (http://distelldeep.ucd.ie/PeptideRanker). This program assigns ranks to peptides based on the probability that they are bioactive, ranging from 0.0 (highly unlikely to be bioactive) to 1.0 (highly likely to be bioactive) (Mooney *et al.*, 2012). Of the 15 unique peptides identified by the bioinformatic analysis of *S. meleagris* collagen in this work, eight of them, with less than 20 residues (Mooney *et al.*, 2012), scored higher than 0.5 (Xia *et al.*, 2022), indicating a high probability of bioactivity: GPPGDQGPQGL (0.84), AGVEGPPGPPGF (0.80), GSQGPTGEKGANGLPGL (0.78), PPGDQGPQGL (0.77), GNAGPKGEPGESGGL (0.63), PGQNGLRGADGIKGEPGL (0.63), KGQPGPG GSADF (0.62), and KGNEGPPGEKGL (0.56). The presence of amino acids, such as glycine, proline, leucine, alanine, phenylalanine, and valine, plays a key role in the antioxidant activities of peptides (Zou *et al.*, 2016). Therefore, it is very likely that the predicted peptides have good antioxidant activity. However, an integrated study of *in vitro* and *in silico* techniques is required to screen their potential antioxidant activity.

Finally, the antioxidant indicator assays assess the ability of a compound (or group of compounds) to interact with a free neutral radical that possesses an unpaired electron in one of its orbitals (McMurry *et al.*, 2012). Free radicals are unstable and highly reactive; their drive to achieve stability through electron scavenging can initiate chain reactions that damage cellular structures, including membranes, lipids, and proteins (De Palma and Clementi, 2014). Given this context, it is possible that pepsin-soluble collagen extracts from jellyfish could donate protons and electrons (through hydrogen atom transfer and single electron transfer systems) to function as antioxidants within a cellular model.

## Conclusion

Pepsin-solubilized collagen was successfully extracted from the mesoglea of cannonball jellyfish. The chemical structure of the obtained collagen suggests the presence of various types of collagens. Conversely, proteomic analysis indicated that the extracted collagen primarily consisted of type IV collagen. Low denaturation temperature detected was probably attributed to low imino acid content in the obtained collagen. The significant antioxidant capacity observed was potentially attributed to the presence of basic, aliphatic, and aromatic amino acids, which contributed to free-radical scavenging, ferric reduction, and oxygen radical absorbance, helping to protect against erythrocyte hemolysis. This study also revealed that antioxidant activity was found to be better than the commercially fish marine collagen. This study provided a theoretical foundation for further exploration into synthesizing antioxidant peptides derived from jellyfish collagen.

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## Author Contributions

Blanca del Sol Villalba-Urquidy developed the formal analysis, the results analysis, and writing—original draft preparation; Josafat Marina Ezquerra-Brauer conceptualized and designed the research; Wilfrido Torres-Arreola

supported the establishment conditions of collagen extraction; Carmen Lizette Del Toro-Sánchez assisted with the appropriate explanation of antioxidant results; Hisila del Carmen Santacruz-Ortega, Isabel Medina and Armando Burgos-Hernández helped with the proper establishment and interpretation of the chemical structural and proteomic analysis. All authors contributed to the writing—original draft preparation. The author responsible for project supervision and funding acquisition was Josafat Marina Ezquerra-Brauer.

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