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Original Article

Comparative antimicrobial and antioxidant activity from collagen peptides from *Seriola rivoliana* fish scales, using shrimp and commercial enzymes

Actividad antimicrobiana y antioxidante de péptidos de colágeno obtenidos a partir de escamas de pescado de *Seriola rivoliana*, utilizando enzimas de camarón y enzimas comerciales

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ABSTRACT
The fish processing industry faces dual challenges: environmental impact and low-profit applications for by-products, emphasizing the need to valorize this waste. In this study, collagen was isolated from *Seriola rivoliana* fish scales. SDS-PAGE results indicated that the purified collagen consisted of two distinct chains (α1- and α2-), consistent with the composition of type I collagen. Fish scale collagen is composed of Gly, Ala and Pro (20 %) amino acids, and exhibits an absorption peak at 230 nm in the UV-Vis spectrum. Collagen was hydrolyzed with 60 and 120 mU of Wobenzym (WE) activity and digestive gland extract from shrimp waste (SE). The resulting collagen-derived peptides from WE showed DPPH scavenging activity, while shrimp-derived peptides did not. Both WE and SE-derived peptides inhibited the growth of marine pathogens (*Vibrio diabolicus*, *Vibrio parahaemolyticus*, and *Photobacterium*) and human pathogens (*Escherichia coli*, *Pseudomonas* spp., and *Salmonella* spp.). However, SE-derived peptides demonstrated stronger inhibitory effects against human pathogens, while WE-derived peptides were more effective against marine pathogens. These results suggest that waste materials, such as scales from the marine fish *S. rivoliana*, have potential as a source of collagen for generating peptides with antioxidant and antimicrobial properties.
Keywords: Collagen, peptides, antioxidant, antimicrobial.

RESUMEN
La industria de procesamiento de pescado enfrenta un doble desafío: el impacto ambiental y el bajo valor de sus subproductos, lo que resalta la necesidad de su valorización. En este estudio, se aisló colágeno a partir de escamas de pescado de *Seriola rivoliana*. El análisis por SDS-PAGE mostró dos cadenas distintas (α1 y α2), consistentes con la composición del colágeno tipo I. Este colágeno está compuesto principalmente por los aminoácidos glicina, alanina y prolina, siendo Pro (20 %) el más abundante, y presentó un pico de absorción a 230 nm en el espectro UV-Vis. El colágeno fue hidrolizado con 60 y 120 mU de actividad de Wobenzym (WE) y extracto de glándula digestiva de camarón (SE). Los péptidos derivados con WE mostraron actividad secuestradora de radicales DPPH, mientras que los generados con SE no presentaron dicha actividad. Sin embargo, ambos tipos de péptidos inhibieron el crecimiento de patógenos marinos (*Vibrio diabolicus*, *Vibrio parahaemolyticus* y *Photobacterium*) y patógenos humanos (*Escherichia coli*, *Pseudomonas spp.* y *Salmonella spp.*), sin embargo, su efecto fue de manera diferenciada. Estos resultados sugieren que las escamas de *S. rivoliana*, tienen potencial como fuente de colágeno para generar péptidos con propiedades antioxidantes y antimicrobianas.
Palabras clave: Colágeno, péptidos, antioxidante, antimicrobiano.

INTRODUCTION
The fishing industry is responsible for producing significant quantities of marine and seafood products, where only 40 % of the catch is destined for human consumption, while more than 60 % (head, skin, trimmings, fins, and viscera) is discarded. As a result, considerable amounts of waste are generated (Chalamaiah *et al*., 2012), leading to serious environmental problems since these wastes have no direct application. The dual problem arising from fish processing industries, including environmental issues and less profitable applications for by-products and waste, has highlighted the need to valorize fish processing waste, while simultaneously contributing to a more sustainable fishing industry.

In this context, research has focused on the search for higher-value products that could be extracted from these fishery by-products (head, skin, trimmings, fins and viscera), such as proteins (proteolytic enzymes, protein hydrolysates, bioactive peptides, collagen, and gelatin), fish oils, and hydroxyapatite (Montero and Gómez-Guillén, 2000). These biomolecules offer numerous applications possibilities, ranging from the food industry to the pharmaceutical, medical, agricultural, and cosmetic industries (Ennaas *et al*., 2015; Ennaas *et al*., 2016).

Collagen Type I is a fibrillar protein composed by a helix structure consisting of two homologous chains (α1) and an additional sequence with slight change in its composition (α2) (Shimizu *et al*., 2001). It is the most abundant protein in mammals representing nearly 30% of total protein in the animal body. Humans consume external collagen, primarily from animal sources, to support joint health, improve skin elasticity, and promote overall tissue repair (Upasen *et al*.,

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2019), since natural collagen production decreases with age. Mammalian collagens, particularly those from pigs and cows, are the most used and popular, but they present challenges due to potential allergic reactions and the risk of transmitting diseases such as bovine spongiform encephalopathy (mad cow disease), ovine and caprine scrapie, and other zoonotic infections (Pati *et al*., 2012).

The major protein constituent of seafood processing waste is collagen, which resembles mammalian collagen (Upasen *et al*., 2019). Collagen from fish has been isolated from skin and scales from several species using acetic acid (Minh-Thuy *et al*., 2014; Upasen *et al*., 2019). It possesses 33 % of glycine as the most abundant amino acid (Minh-Thuy *et al*., 2014; Huang *et al*., 2016) and an absorption at 230 nm by UV-Vis spectra (Zhang *et al*., 2011). Acetic acid extraction of collagen, also known as acid soluble collagen (ASC), displays a yield range of 0.69 - 6.20 % (Minh-Thuy *et al*., 2014; Chen *et al*., 2016). However, increasing yield of 1.06 – 9.5 % (Chen *et al*., 2016; Upasen *et al*., 2019) has been obtained by acetic acid extraction followed by an enzymatic hydrolysis with pepsin without affecting their physicochemical properties (Ali *et al*., 2018). Collagen yields depended on fish species, habitat, environment as well as the source of collagen (Minh-Thuy *et al*., 2014). Fish collagen possesses similar characteristics to porcine collagen, and thus, may be considered as an alternative to mammalian collagen (Pati *et al*., 2012).

Collagen and its hydrolysate (gelatin) protein contain, encrypted within their primary structure, a wide range of bioactive peptides which may be released by enzymatic hydrolysis (Ennaas *et al*., 2016). The hydrolysate gelatin products have been recognized as GRAS (Generally Recognized as Safe) by the US Food and Drug Administration (FDA) (Lukin *et al*., 2022). Bioactive peptides from fish collagen hydrolysis allowed the identification of an antibacterial peptide active against *Listeria innocua* HPB13 and *Escherichia coli* MC4100 (Ennaas *et al*., 2015) and *Staphylococcus aureus* potentially through a carpet mechanism (Ennaas *et al*., 2016). Antioxidant activity (Wang *et al*., 2013) and functional (solubility, foaming, and emulsifying ability) properties have been described from peptide fractions obtained from by-products derived from the collagen hydrolysate of a mixture of different fish species (Ennaas *et al*., 2016; Zamorano-Apodaca *et al*., 2020). Thus, converting fish and seafood waste into valuable compounds with nutritional and functional properties offers a new alternative to mammal-based products.

Therefore, the aim of this study was to isolate collagen from fish scales from a commercial fish species, *Seriola rivoliana*, a fast-growing species inhabiting subtropical oceans, which has emerged for aquaculture diversification around the world (Jerez, 2013). Collagen from scales was hydrolyzed with a commercial enzyme and with an extract from hepatopancreas from shrimp *Litopenaeus vannamei*, which is an important commercial species which accounts for 90 % of the global aquaculture shrimp production without hepatopancreas. The hepatopancreas, a waste from aquaculture, is a well-known source of enzymes, which actively hydrolyze protein, lipids and carbohydrates (Rojo-Arreola *et al*., 2019). The antimicrobial and antioxidant activity were evaluated for collagen and for the collagen derived peptides.

MATERIAL AND METHODS

Fish

Fish skin with scales from *Seriola rivoliana* was obtained from local fish markets in La Paz, Baja California Sur, México. The fish skins were transported to the Biochemistry Laboratory at Centro de Investigaciones Biologicas del Noroeste S.C. The scales were removed from the fish skin manually, washed with cold water to remove residues and salt, and then dried overnight at room temperature.

Preparation of scale collagen

Collagen from scales were isolated according to the methodology describe by Pati *et al*. (2012). Briefly, scales (~ 60 g) from *S. rivoliana* were treated with 0.1 M NaOH to remove non-collagenous proteins and pigments for 72 h with constant agitation at 4 °C. Afterwards, scales were washed thoroughly with distilled water. Then, collagen was extracted with 0.5 M acetic acid for 72 h with constant agitation at 4 °C, and the extract was centrifuged at 10, 000 rpm for 1 h at 4 °C in a Sorvall RC 6+ centrifuge (Thermo Scientific). The supernatant containing the soluble collagen was isolated by salting out by adding NaCl to a final concentration of 2.3 M. The addition of NaCl was performed with constant agitation for 1 h. The resulting solution was centrifuged at 10, 000 rpm for 1h at 4° C in a Sorvall RC 6+ centrifuge (Thermo Scientific). The supernatant was discarded and the pellet containing the precipitated collagen was re-solubilized in 0.5 M acetic acid. The solubilized collagen was dialyzed (molecular weight cut off range 12 - 14 kDa/ 45 mm / 6.4 mL/cm- Spectrum) against 0.1 M acetic acid for 24 h with constant agitation at 4 °C. Then, a second dialysis was performed against distilled water for 24 h with constant agitation at 4 °C. Finally, the dialyzed collagen was recovered and freeze- dried for further analysis. These processes were carried out by triplicate.

Amino acid analysis

Collagen samples were hydrolyzed under reduced pressure in 6 M HCl at 110°C for 22 h, and the hydrolysates were analyzed by an amino acid analyzer HP1100 (Agilent, USA) (Gratzfeld-Huesgen, 1999).

UV-Vis Spectra

The UV-Vis absorption spectrum of collagen from *S. rivoliana* scales was recorded using a Synergy microplate reader (Agilent Technologies) in the range of 200 – 400 nm (Zhang *et al*., 2011). For this analysis, 1 mg of collagen was dissolved in 250 µL of 0.1 M McIlvaine buffer (pH 6.0). McIlvaine buffer was used as blank.

SDS-PAGE

Isolated collagen from *S. rivoliana* fish scales was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis

(SDS-PAGE) according to Laemmli (1970). Thirty micrograms of protein were mixed with 4x sample buffer (5 M Tris–HCl pH 6.8, 20 % glycerol, 10 % SDS, 10 % β-mercaptoethanol and 0.05 % bromophenol blue). Samples were boiled for 10 min and then loaded into a 10 % (collagen) or 15 % (collagen derived peptides) polyacrylamide gel. Broad range molecular weight standard (Bio-Rad, 1610317, California, USA) was loaded into the gel. Electrophoresis was done on a constant current of 15 mA per gel at 4 °C, using a vertical electrophoresis unit (Hoefer, Inc Hoefer SE600). After electrophoresis, the gels were stained for 2 h with staining solution (0.05 % (w/v) Coomassie Brilliant Blue R-250, 7 % (v/v) acetic acid, 40 % (v/v) methanol). Proteins were revealed by soaking for 2 h in destaining solution (7 % (v/v) acetic acid and 40 % (v/v) methanol). Gel proteins were analyzed using a gel imager (Chemi Doc XRS, Bio-Rad, California, USA).

Protein quantification

The protein concentration in the supernatant solutions was assayed by the Bradford method (Bradford, 1976) and compared with a standard, bovine serum albumin (Sigma, B-4287, St. Louis, MO).

Hydrolysis of the collagen peptides

To generate collagen peptides two different sources of enzymes were used, Wobenzym (WE) (Rivera-Pérez *et al*., 2023), a commercial mixture of commercial enzymes of mixed origin, animal and plant (Table S1), and a shrimp enzyme extract (SE). To standardize the units of activity for the hydrolysis experiment using each set of enzymes, total proteolytic activity was measured using 0.5 % azocasein as substrate in 0.1 mM McIlvaine buffer, pH 6.0 at 25 °C. The mixture was incubated for 10 min, following the methodology described by García-Carreño *et al*. (1993). Total proteinase can be expressed as the change in absorbance per minute per milligram of protein of the enzyme extract used in the assays or as international units IU of µmol of substrate cleaved per minute, based on the substrate extinction coefficient.

Collagen hydrolysis was performed with WE and SE, respectively. Reaction mixture contained 1 mg (dried weight), 0.1 M McIlvaine buffer pH 6.0, and enzyme (WE or SE) containing 60 or 120 mU. The mixture was incubated at 37 °C with constant agitation (300 rpm) for 1 h. The enzymes were inactivated at 95 °C for 15 min and stored at - 20 °C for further analysis. Controls without enzymes and with both enzyme mixtures were included.

Table S1. Wobenzym mixture of enzymes composition with origin and dried mass.
Tabla S1. Composición enzimática de la mezcla Wobenzym con su origen y masa seca.

Origin Enzyme	Enzyme	Mass (mg)
Sus scorfa (pancreas)	Pancreatin	300
Carica papaya	Papain	180
Ananas comosus	Bromelain	135
Sus scorfa	Trypsin	72
Bos taurus	Chymotrypsin	3
Sophora japonica	Rutoside trihydrate	150

Antioxidant activity

Antioxidant activity was measured by scavenging activity against free radicals using 2,2-diphenyl1-picrylhydrazyle (DPPH) radicals (Prieto, 2012). The DPPH assay was performed by mixing 180 µL of the DPPH solution and 20 µL of the sample. The mixture was incubated for 30 min in the dark at room temperature, and absorbance was measured at 515 nm using a spectrophotometer (VERSAmax microplate reader, Molecular Devices). All the experiments were performed in triplicate, and the scavenging activity (%) was calculated as 100 % x (Ai-Aj)/Ac, where Ai is the absorbance of the samples + DPPH, Aj is the absorbance of the sample + DPPH solvent, and Ac is the absorbance of the DPPH solvent + DPPH.

Bacterial strains

Two bacterial strains CIBGEN-002 and CIBGEN-003 were used in this study, which were previously isolated from the sediment of shrimp ponds with acute hepatopancreatic necrosis disease (AHPND) and identified by whole-genome as *Vibrio diabolicus* and *Vibrio parahaemolyticus* respectively. Bacterial numbers were estimated in colony-forming units (CFU) at 10^8 CFU per mL in *V. diabolicus* and 10^7 CFU per mL in *V. parahaemolyticus* after serial dilution in 2.5 % NaCl and plating on TSA. Human pathogens were isolated from the wastewater treatment plant in La Paz by the Microbiology Lab from CIBNOR, and used in this study (Table 1)

Antimicrobial activity

Antimicrobial activities of collagen peptides derived from scales of *S. rivoliana* were assessed by spectrophotometry, measuring their effect on bacterial growth (Table 1). Briefly, 50 µL of culture at 0.1 OD and 25 µL of the sample (100 µg protein of collagen peptides) were added to 165 µL of Trypto-Casein Soy Broth (TSB) supplemented with 2.5 % NaCl. The effect on the bacterial growth was monitored every 30 min preceded by agitation for 24 h at 35 °C in a 96-well microplate reader (Multiskan GO). Positive (bacteria alone) and negative (media alone) controls were included.

Table 1. Strains used for this study.
Tabla 1. Cepas utilizadas en este estudio.

Strain	Growth media	Reference/ATCC
Vibrio diabolicus (CIBGEN 002)	TSB	Veyrand-Quiros *et al*., 2020
Vibrio parahaemolyticus (CIBGEN 003)	TSB	Veyrand-Quiros *et al*., 2020
Photobacterium spp.	TSB	Veyrand-Quiros *et al*., 2020
Escherichia coli	LB	25922
Pseudomonas spp.	LB	15442
Salmonella spp.	LB	35664

Statistical analysis

The effects of collagen-derived peptides on bacterial growth were analyzed for significance using a one-way ANOVA. The Shapiro-Wilk test was used to assess normality, and a Tukey post-hoc test identified differences among means at a significance level of $P > 0.05$. All statistical analyses were conducted using the R software.

Ethical Statement

The authors followed all applicable international, national, and institutional guidelines for the care and use of fish.

RESULTS AND DISCUSSION

Collagen yield and protein concentration

Collagen was extracted from scales of *Seriola rivoliana* using three independent batches. The mean soluble protein concentration after the collagen isolation process was 0.040 ± 0.014 mg/mL. Although this concentration and the final collagen yield, calculated as 0.083 % ± 0.06 based on the initial mass of the scales and the final dry mass of collagen extract appear low, comparable studies indicate variable yields across fish species and extraction methods. For example, acidic extraction has been reported to produce yields ranging from as low as 0.05 % in *Thunnus obesus* (Ahmed *et al*., 2019), 0.73 % in *Chanos chanos* (Wahyu and Widjanarko, 2018) to 1.0 % in *Scarus sordidus* (Jaziri *et al*., 2023) among marine species. Furthermore, modifications in the extraction conditions, particularly pH and temperature, can significantly enhance recovery. Under optimized conditions, yields have reached 27.5 % from fish scales of *Larimichthys crocea* (Feng *et al*., 2020), and up to 39 % from scales of the freshwater species *Oreochromis aureus* (Hernández-Ruiz *et al.,* 2023). These values contrast with higher efficiencies obtained through alternative extraction techniques. For example, pepsin-soluble collagen extraction in *Sciaenops ocellatus* yielded 4.42 % (Chen *et al*., 2016), while ethanol-hexane extraction in *Oreochromis* spp. achieve around 49.42% (Huang *et al*., 2016). The comparative lower yield observed in acetic-soluble extraction method from scales may be attributed to a higher degree of molecular cross-linking among collagen molecules in scales compared to skin (Feng *et al*., 2020), which can yield over 50 % (Huang *et al*., 2016). Additionally, collagen yield is influenced by factors such as fish species, habitat, environmental conditions, and the specific collagen source (Minh-Thuy *et al*., 2014).

Sub-unit composition and UV-Vis Spectra

The SDS-PAGE analysis of collagen from scales (Fig. 1A) displays the protein profile of a type I collagen (lane 1), comprised by distinct bands that typically represent the α1- and α2-chains (~120 kDa) with the cross-linked dimers (β-chain, ~ 240 kDa) and small amounts of trimers (γ -chains). This electrophoretic pattern was similar to the reported for bovine tendon (Chen *et al*., 2016) and those for marine scales from *Thunnus obsesus* (Ahmed *et al*., 2019) and *Miichthys miiuy* (Li *et al*., 2018). The UV absorption spectra of collagen extracted from fish scales is shown in Fig. 1B. The spectra were recorded over a wavelength range of 200 to 400 nm for three independent batches, labeled B1, B2, and B3, represented by dashed, dotted, and solid red lines, respectively. All three samples show a prominent absorption peak around 220 - 230 nm, which is typical for collagen and corresponds to the C=O, –COOH, and $CONH_2$ groups in the collagen polypepti-

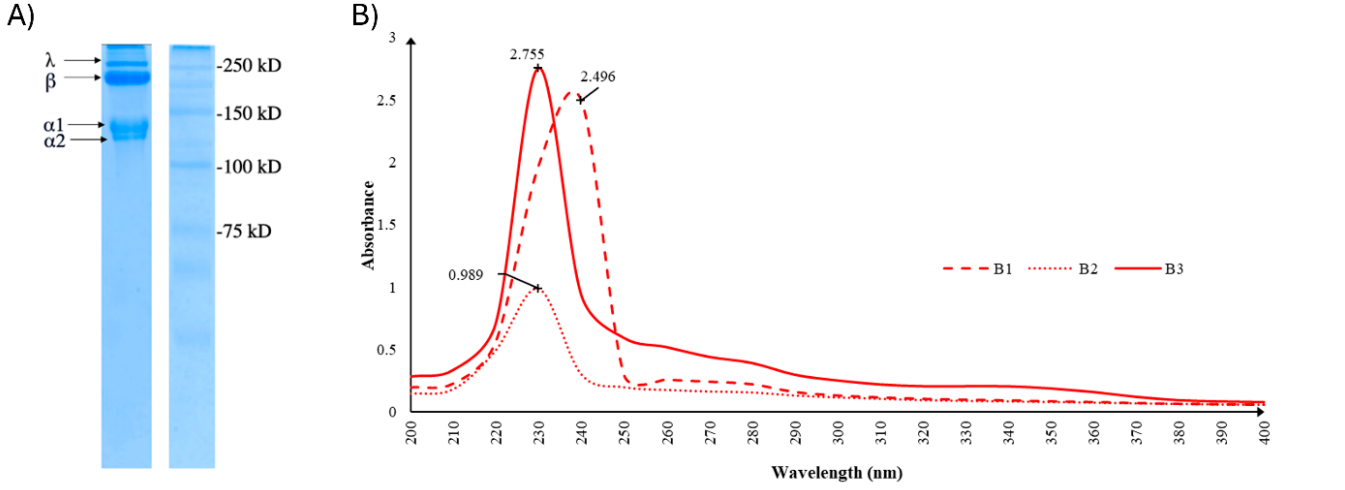

Figure 1. Collagen (30 µg) from *Seriola rivoliana* fish scales. A) SDS-PAGE gel electrophoresis (10 %) of extracted collagen. Lane 1, the extracted collagen; Lane 2, high molecular weight marker (Precision Plus Protein Dual Color Standards – BIO-RAD). Arrows indicate chains of collagen type I. The image is representative of three independent batches. Gel was stained with Coomassie Brilliant Blue R250. B) UV absorption spectra of collagen extracted from scales from *S. rivoliana.* Absorbance is expressed in arbitrary units (a.u.). B1-3: independent batches.

Figura 1. Colágeno (30 µg) de escamas de pescado de *Seriola rivoliana*. A) Electroforesis en gel SDS-PAGE (10 %) del colágeno extraído. Carril 1, colágeno extraído; Carril 2, marcador de peso molecular alto (Precision Plus Protein Dual Color Standards – BIO-RAD). Las flechas indican las cadenas del colágeno tipo I. La imagen es representativa de tres lotes independientes. El gel fue teñido con Azul Brillante de Coomassie R250. B) Espectros de absorción UV del colágeno extraído de escamas de *S. rivoliana*. La absorbancia está en unidades arbitrarias (a.u.). B1-3: lotes independientes.

des chains (Yan *et al*., 2008; Hernández-Ruiz *et al*., 2023). The peak absorbance values differ slightly among the batches: B1 reaches a maximum of 2.755, B2 has a peak of 0.989, and B3 peaks at 2.496. These variations suggest differences in the purity across the batches. After 250 nm, the absorbance rapidly decreases, remaining relatively low and stable from 300 nm onward, with no significant peaks or features. This profile confirms the presence of collagen, as proteins generally do not absorb strongly beyond 300 nm, which indicates minimal contamination with other UV-absorbing compounds. Similar results have been described in fish skin (Atef *et al*., 2021) and scales (Hernández-Ruiz *et al*., 2023) from marine and freshwater species.

Amino acid composition

Fish collagen has been recognized for its diverse amino acid composition (Chinh *et al*., 2019). The percentages of amino acids in *S. rivoliana* were obtained after acidic hydrolysis of the protein for 24 h and compared to results from other fish scale collagens (Table 2). Proline (Pro) content was higher in *S. rivoliana* (20.61 %) than in *Pagrus major* (red sea bream) (10.19 %), *Cololabis saira* (Pacific saury) (9.1 %), and porcine collagen (12.5 %). It is well known that proline content in collagen significantly influences its structural and functional properties (Shoulders and Raines, 2009), providing advantages in terms of structural stability and thermal resistance. Proline contributes to the stabilization of the collagen triple helix through its rigid cyclic structure, which restricts conformational flexibility of the polypeptide chain. This rigidity enhances intermolecular hydrogen bonding and hydrophobic interactions, resulting in improved structural stability. In addition, the high proline content increases the denaturation temperature of collagen, thereby conferring greater thermal resistance (Shoulders *et al*., 2009). Also, peptides containing Pro and Hyp, exhibit bioactivity since are more resistant to the action of peptidases in the digestive system (Ucak *et al*., 2021). These characteristics make it particularly beneficial for biomedical and cosmetic applications.

Hydrolysis of the collagen peptides

Collagen hydrolysates were produced with two different sources of enzymes, a commercial mixture of enzymes (Wobenzym; WE) and an enzymatic extract from the digestive gland (shrimp enzyme; SE) of the white-leg shrimp *Litopenaeus vannamei*. The hydrolysis conditions were standardized before collagen hydrolysis (pH and time of hydrolysis). Collagen from scales was hydrolyzed with two different units of activities, 60 and 120 mU of WE and SE, respectively using 0.1 M McIlvaine buffer pH 6.0 at 37 °C during 1 h (Fig. 2). The hydrolysis patterns show differences between the untreated collagen (Fig. 2A) and the hydrolyzed samples (Fig. 2B). The hydrolyzed samples show additional lower-molecular-weight bands, indicating collagen breakdown products. As the enzyme activity increases from 60 to 120 mU, the extent of hydrolysis becomes more pronounced, with clearer fragmentation and additional lower-molecular-weight bands. WE, which contains serine- and cysteine-type proteases, and SE, which includes a broader range of proteases (serine, cysteine, aspartic, and metalloproteases), may generate a distinct banding pattern due to differences in cleavage specificity and efficiency in collagen hydrolysis.

Table 2. Amino acid composition (%) of fish scale collagens from marine species compared to porcine collagen.
Tabla 2. Composición de aminoácidos (%) de colágeno de escamas de pescado de especies marinas comparadas con colágeno porcino.

Amino acid	*Seriola rivoliana* (this study)	*Pagrus major**	*Cololabis saira**	Porcine collagen*
Glu	6.03	7.97	7.08	7.5
Asp	9.67	5.3	6.14	4.82
Ser	3.20	3.79	5.18	2.62
His	0.00	1.07	1.31	0.88
Gly	24.13	35.31	33.54	36.7
Thr	2.90	2.71	3.02	0.66
Ala	12.20	13.65	11.59	12.13
Arg	7.92	5.14	4.81	5.05
Tyr	0.69	0.59	1.14	0.53
Val	2.31	2.74	3.43	3.33
Phe	3.73	1.6	1.77	1.5
Ile	1.42	1.2	1.62	1.29
Leu	2.40	2.32	2.99	2.58
Lys	2.78	3.35	3.34	2.92
Pro	20.61	10.19	9.1	12.5

*Mori *et al*., 2013

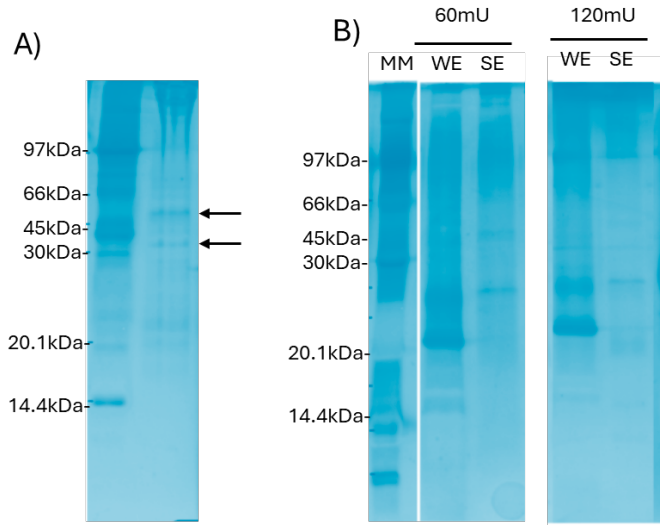

Figure 2. Hydrolysis of collagen from fish scales. A) Collagen untreated, B) Collagen hydrolyzed (30 µg) with Wobenzym (WE) and shrimp enzymes (SE), using 60 or 120 mU of activity. SDS-PAGE 15 % for gel A, and 12 % for gel B. Arrows indicate collagen chains from fish scales.
Figura 2. Hidrólisis del colágeno de escamas de pescado. A) Colágeno sin tratar, B) Colágeno hidrolizado (30 µg) con Wobenzym (WE) y enzimas de camarón (SE), utilizando 60 o 120 mU de actividad. SDS-PAGE al 15 % en gel A y 12 % en gel B. Las flechas indican las cadenas de colágeno de las escamas de pescado.

Other studies have demonstrated a higher degree of collagen hydrolysis when using enzyme mixtures (e.g. Flavourzyme, Protamex, etc) which release low-molecular-weight products (<37 kDa) (Atef *et al*., 2021; Hernández-Ruiz *et al*., 2023; Xu *et al*., 2024). However, the most used enzymes for collagen hydrolysis are pepsin and papain, which typically produce hydrolyzed proteins below 75 kDa (Gonzalez-Serrano *et al*., 2022). In this study, we compared a commercial enzyme mixture, which was previously proved to generate bioactive peptides in abalone viscera (Rivera-Perez *et al*., 2023), with an enzyme extract derived from shrimp fishery waste, which is known to contain stable trypsin and chymotrypsin activities (Rojo-Arreola *et al*., 2019). Both enzyme sources were effective in hydrolyzing *S. rivoliana* collagen, generating peptides smaller than 30 kDa. This suggests that shrimp enzymes derived from the digestive gland could be effectively used for collagen hydrolysis.

Antioxidant activity

The antioxidant activity of collagen and collagen peptides are shown in Fig. 3. The results show that ascorbic acid (positive control) exhibits the highest antioxidant activity (89 % ± 1.0). Isolated collagen shows a lower antioxidant activity (47.94 % ± 34.7) compared to the positive control. For the enzyme-treated samples, WE hydrolysis significantly enhanced the antioxidant activity of collagen in a dose-dependent manner. The samples hydrolyzed with 120 mU of WE exhibit higher antioxidant activity (83.24 % ± 8.38) than those hydrolyzed with 60 mU (50.79 % ±14.07), while SE-derived peptides showed no activity. Comparable effects of enzymatic hydrolysis have been reported in other fish species: silver carp scales treated with alcalase and flavourzyme reached 93.4 % antioxidant activity (Xu *et al*., 2024), whereas lower activities were observed for redlip croaker scales hydrolyzed with neutrase (30.97 %; Wang *et al*., 2020) and miiuy croaker scales digested with trypsin (~ 50 %; Li *et al*., 2018). These differences suggest that both the enzyme type and hydrolysis conditions strongly influence antioxidant potential. The higher activity observed for WE hydrolysates may be explained by the exposure of amino acids such as glycine, glutamic acid, alanine, and aspartic acid, which promote free radical stabilization (Zamorano-Apodaca *et al*., 2020). Furthermore, enzymatic reactions coupled with physical aggregation likely facilitated the formation of larger yet more concentrated peptide aggregates, increasing the exposure of hydrophobic groups and thereby enhancing antioxidant capacity (Xu *et al*., 2024).

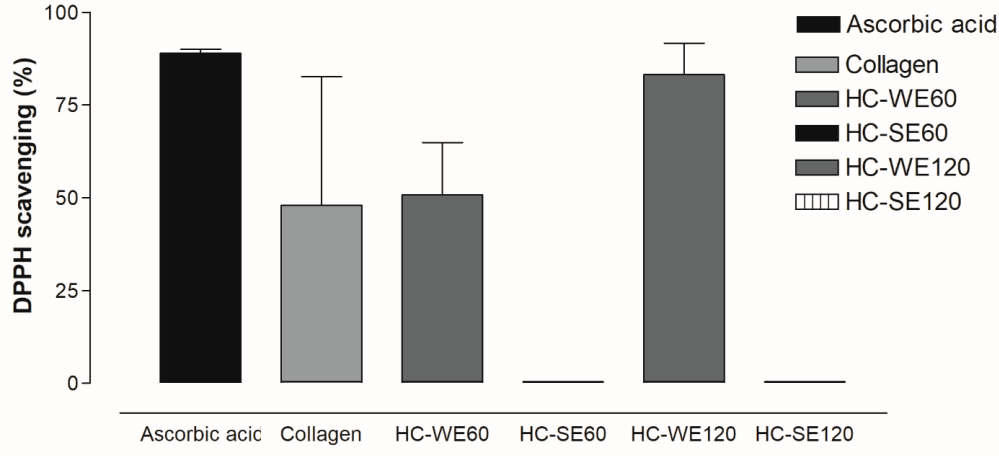

Figure 3. Percentage of DPPH scavenged by isolated collagen, hydrolyzed collagen and ascorbic acid (positive control) from all biological replicates (B1, B2, B3) in each hydrolysis condition (using WE and SE at 60 and 120 mU of activity). HC: hydrolyzed collagen, WE: Wobenzym, SE: Shrimp enzyme.
Figura 3. Porcentaje de DPPH neutralizado por colágeno aislado, colágeno hidrolizado y ácido ascórbico (control positivo) a partir de todas las réplicas biológicas (B1, B2, B3) en cada una de las condiciones de hidrólisis (utilizando WE y SE a 60 y 120 mU de actividad). HC: colágeno hidrolizado, WE: Wobenzym, SE: enzima de camarón.

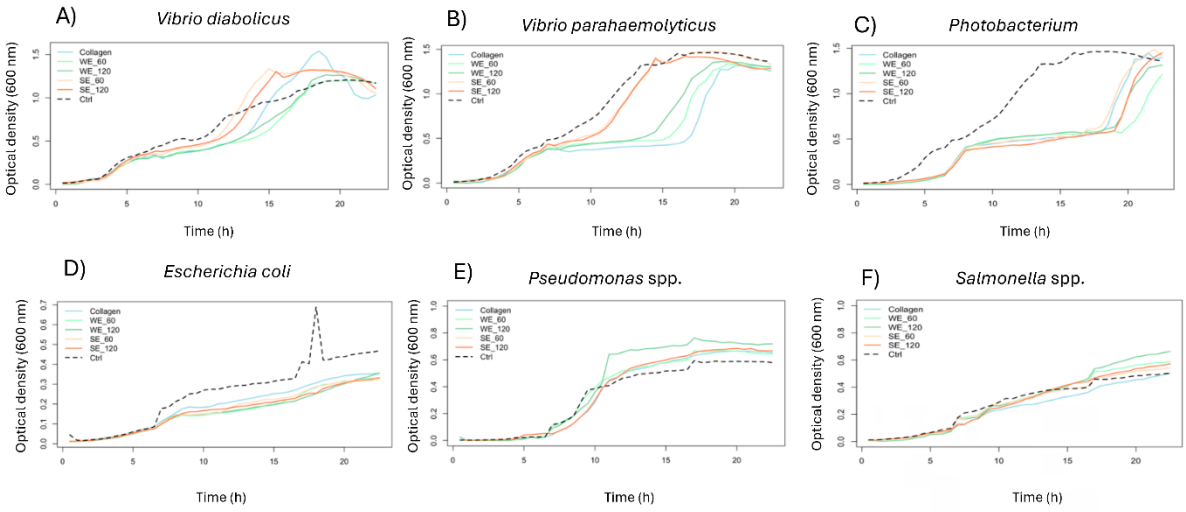

Figure 4. Antimicrobial activity of fish scale collagen, hydrolyzed collagen treated with Wobenzyme, and hydrolyzed collagen treated with shrimp enzyme against six selected bacterial strains, monitored by growth curves at 600 nm. A) *Vibrio diabolicus*, B) *Vibrio parahaemolyticus*, C) *Photobacterium*, D) *Escherichia coli*, E) *Pseudomonas* spp., F) *Salmonella* spp.
Figura 4. Actividad antimicrobiana del colágeno de escamas de pescado, colágeno hidrolizado tratado con Wobenzym y colágeno hidrolizado tratado con enzima de camarón frente a seis cepas bacterianas seleccionadas, monitoreada mediante curvas de crecimiento a 600 nm. A) *Vibrio diabolicus*, B) *Vibrio parahaemolyticus*, C) *Photobacterium*, D) *Escherichia coli*, E) *Pseudomonas spp.*, F) *Salmonella spp.*

Antimicrobial activity

Fish scale collagen (control) established baseline growth rates for the tested pathogens (Fig. 4A–F), with values ranging between 0.30 – 0.75 h^{-1} depending on the species (e.g., *E. coli* μ = 0.30, *Pseudomonas* spp. μ = 0.75, Table 3). When collagen was hydrolyzed with Wobenzym, the resulting peptides generally reduced bacterial growth compared to the control. In particular, WE_120 consistently lowered growth rates in marine pathogens, reaching μ = 0.46 h^{-1} *in V. diabolicus* and μ = 0.50 h^{-1} in *Photobacterium*, which were markedly lower than the corresponding controls. However, in *Pseudomonas* spp. WE treatments strongly enhanced growth, with μ values rising to 1.10 h^{-1} under WE_120, suggesting a species-specific utilization of WE-derived peptides.

Treatments with shrimp enzyme extracts (SE) elicited more variable responses across species (Fig. 4). For *V. diabolicus* and *Photobacterium*, SE_120 reduced growth (μ ≈ 0.48–0.62 h^{-1}), supporting an inhibitory effect. In contrast, in enteric bacteria such as *E. coli*, SE_120 markedly increased growth (μ = 0.48 h^{-1} vs. 0.30 in collagen), while in *Salmonella* the effect was neutral to slightly stimulatory. These differences suggest that shrimp-derived peptides may act as inhibitory compounds for some marine pathogens but provide accessible nutrients for enteric species.

Overall, the results indicate that enzyme source and hydrolysis duration are critical determinants of antimicrobial activity. Wobenzym-derived peptides (especially at 120 mU) showed more consistent inhibitory effects across vibrios and Photobacterium, while shrimp enzyme-derived peptides displayed species-specific outcomes: inhibitory against some marine bacteria, but occasionally stimulatory in enteric pathogens such as *E. coli* and *Salmonella*. This variability likely reflects differences in the peptide profiles generated by the distinct protease systems, with certain hydrolysates producing bioactive antimicrobial peptides, while others yield substrates that support bacterial growth.

This study further indicate that peptides generated from WE and SE exhibit distinct antibacterial activities, likely

Table 3. Microbial growth responses (μ, h^{-1}) of representative strains after exposure to collagen hydrolysates.
Tabla 3. Respuestas de crecimiento microbiano (μ, h^{-1}) de cepas representativas tras la exposición a hidrolizados de colágeno.

Strain	Collagen	WE_60	WE_120	SE_60	SE_120	Ctrl
V. diabolicus	0.590	0.569	0.537	0.505	0.483	0.537
V. parahaemolyticus	0.590	0.569	0.537	0.611	0.642	0.652
Photobacterium	0.611	0.537	0.505	0.590	0.621	0.642
E. coli	0.295	0.328	0.339	0.317	0.483	0.404
***Pseudomonas* spp.**	0.747	0.948	1.097	0.898	0.948	0.747
***Salmonella* spp.**	0.490	0.646	0.697	0.594	0.646	0.542

influenced by differences in peptide size and composition, which in turn may modulate their functional properties (Poorna *et al*., 2012). Hydrolyzed peptides from the skin and scales of various fish species have demonstrated effectiveness against several bacterial strains, including *Aeromonas hydrophila* (Ulzanah *et al*., 2023), *E. coli*, *Listeria innocua*, and *Salmonella* (Atef *et al*., 2021; Ennaas *et al*., 2015) and *Flavobacterium psychrophilum* and *Renibacterium salmoninarum* (Wald *et al*., 2016). However, the antibacterial potency can vary based on the hydrolysis method and the specific peptides produced, as observed in studies using different enzymes to hydrolyze collagen from fish skin (Atef *et al*., 2021).

The findings of this study highlight the potential of collagen and its hydrolysates from *Seriola rivoliana* scales as promising sources of bioactive peptides with antioxidant and antimicrobial properties. Future research should focus on the purification and characterization of specific peptide fractions to identify the sequences responsible for these activities and to elucidate their underlying mechanisms of action. In addition, in vivo studies and bioavailability assessments are needed to confirm the functional effects observed in vitro. From an applied perspective, scaling up the extraction and hydrolysis processes, while optimizing cost-effectiveness and sustainability, could enable the development of collagen-derived ingredients for biomedical, nutraceutical, and cosmetic applications. Finally, exploring the use of other enzymatic systems or combined hydrolysis strategies may further enhance the bioactivity of collagen peptides, broadening their potential for industrial and therapeutic utilization.

CONCLUSIONS

Type I collagen was successfully extracted from the scales of *Seriola rivoliana* with a yield of 0.083 % ± 0.06 and a soluble protein concentration of 0.040 ± 0.014 mg/mL. Hydrolysis with Wobenzym (WE) significantly enhanced antioxidant activity in a dose-dependent manner, reaching 83.24 % ± 8.38 at 120 mU, a value close to the positive control (ascorbic acid, 89 % ± 1.0), while shrimp enzyme (SE)-derived peptides showed negligible antioxidant effects. Antimicrobial assays demonstrated that WE-derived peptides reduced growth rates of marine pathogens such as *Vibrio diabolicus* ($\mu = 0.46$ h^{-1} vs. 0.75 h^{-1} in controls), whereas SE-derived peptides were more effective against human enteric pathogens, reducing *E. coli* growth to $\mu = 0.48$ h^{-1} compared to 0.30 h^{-1} in controls.

This study introduces an innovative approach by comparing a commercial enzyme mixture with an enzymatic extract obtained from shrimp fishery waste, a low-cost and sustainable resource. The use of SE represents a novel valorization strategy for fishery by-products, offering an alternative source of bioactive peptides with targeted antimicrobial activity. These findings highlight the potential of collagen hydrolysates from *S. rivoliana* scales for application in the fish-processing industry, where they could be developed into natural antioxidant and antimicrobial agents for food preservation, nutraceuticals, and biomedical formulations.

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