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Research Article

Antimicrobial peptide fractions obtained from pen shell *Atrina* (*Servatrina*) *maura* viscera through enzymatic hydrolysis and their potential applications in aquaculture

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ABSTRACT. Marine-derived proteins represent an important source of bioactive peptides with antimicrobial potential. This study evaluated peptide fractions obtained from the viscera of the pen shell *Atrina* (*Servatrina*) *maura* through controlled enzymatic hydrolysis using a multi-enzyme system. Enzymatic hydrolysis increased soluble protein content by 1.48-fold compared to crude extracts, indicating efficient proteolytic processing. Size-exclusion chromatography revealed distinct peptide populations with molecular weights below 29 kDa, which were further characterized by SDS-PAGE analysis. Selected peptide fractions showed antimicrobial activity against Gram-negative bacteria, including *Enterobacter aerogenes*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Shigella flexneri*, and *Shigella sonnei*. Dynamic growth inhibition assays demonstrated concentration-dependent effects, with up to 80% reduction in bacterial growth at 200 µg mL^{-1}. Fractions enriched in lower molecular weight peptides exhibited stronger inhibitory activity, suggesting a relationship between peptide size and antimicrobial effectiveness. These results indicate that enzymatically derived peptide fractions from *A. maura* viscera represent a promising source of antimicrobial biomolecules with potential applications in biotechnology and aquaculture.

Keywords: pen shell viscera; enzymatic hydrolysis; marine bioactive; compounds; antimicrobial peptide; proteins; aquaculture

INTRODUCTION

The escalating prevalence of multidrug-resistant (MDR) pathogens poses a major global challenge to public health, food security, and the sustainability of aquaculture (Kohl 2019). The extensive and often indiscriminate use of conventional antibiotics has accelerated the emergence of resistant microbial strains, reducing the effectiveness of existing treatments and prompting the search for alternative antimicrobial strategies that are both effective and environmentally sustainable (Chen et al. 2024). Among the promising alternatives, bioactive peptides derived from natural protein sources have gained increasing attention.

Marine ecosystems, characterized by high biodiversity and strong selective pressures, have emerged as valuable reservoirs of bioactive compounds (Chen et al. 2024b). In particular, antimicrobial peptides (AMPs) are small, naturally occurring molecules that constitute a key component of innate immunity across a wide range of aquatic organisms, including mollusks, crustaceans, and fish (Zhao et al. 2010, Liao et al. 2013). Their broad-spectrum antimicrobial activity,

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rapid mode of action, and lower propensity for inducing resistance make them attractive candidates for applications in aquaculture and food safety (Zasloff 2019, Cunha & Pintado 2022).

AMPs typically possess a net positive charge conferred by lysine and arginine residues and display amphipathic structures that facilitate efficient interaction with microbial membranes (Chen et al. 2024b). These structural characteristics enable membrane destabilization via physical mechanisms, thereby reducing the likelihood of resistance development compared with conventional antibiotics (Zasloff 2019). In aquatic environments, AMPs also exhibit notable stability under high salinity conditions, supporting their functional relevance in marine systems (Barroso et al. 2021).

Beyond naturally occurring AMPs, enzymatic hydrolysis of marine proteins represents an effective strategy for releasing encrypted bioactive peptide sequences from precursor macromolecules. The biological activity of these hydrolysates is strongly influenced by molecular size distribution, degree of hydrolysis, and amino acid composition, emphasizing the importance of structure-function relationships in determining antimicrobial efficacy. Mollusks, due to their constant exposure to diverse microbial communities, possess efficient innate immune defenses and represent a rich yet underexplored source of antimicrobial peptides. Most characterized molluscan AMPs are cationic and cysteine-rich peptides, classified into four main families, defensins, mytilins, myticins, and mytimycins, based on their amino acid sequences and disulfide bond patterns (Adhya et al. 2012, Zhang et al. 2015). These peptide families exhibit diverse antimicrobial spectra and functional properties, including antibacterial, antifungal, antiviral, and immunomodulatory activities (Rey-Campos et al. 2021). Despite this diversity, only a limited number of molluscan AMPs have been fully characterized, and their broader biotechnological applications remain insufficiently explored.

In aquaculture systems, the search for alternative antimicrobial agents is particularly pressing, as disease outbreaks are often controlled with antibiotics, which contribute to antimicrobial resistance, environmental contamination, and food safety concerns (Lafferty et al. 2015). Approximately 350 AMPs have been identified from aquatic invertebrates, highlighting their potential as therapeutic agents, feed additives, or components of integrated disease management strategies (Garcia-Beltran et al. 2023).

In addition to their functional relevance, marine processing by-products represent an abundant and underutilized source of protein-rich biomass. Seafood processing generates substantial amounts of organic waste, including viscera, which contain proteins that may serve as precursors of bioactive peptides (Ngandjui et al. 2024). Controlled enzymatic hydrolysis of such biomacromolecular resources yields peptide-rich fractions with potential antimicrobial properties, thereby contributing to a more sustainable exploitation of marine resources.

The pen shell *Atrina* (*Servatrina*) *maura*, a bivalve mollusk native to the Gulf of California, is commercially harvested, and its viscera, accounting for approximately 15-25% of the animal's wet weight, are typically discarded during processing. In Mexico, an annual harvest of approximately 490 t of *A*. (*S*.) *maura* generates an estimated 73.65 to 122.75 t of visceral waste (CONAPESCA 2025). These protein-rich tissues represent a promising substrate for the production of bioactive peptide fractions via enzymatic hydrolysis.

Therefore, the objective of this study was to obtain and characterize peptide-rich hydrolysates derived from the viscera of *A.* (*S.*) *maura* and to evaluate their antimicrobial activity. By examining the relationship between molecular characteristics and biological function, this work contributes to the understanding of marine-derived biomacromolecules as potential sources of antimicrobial agents.

MATERIALS AND METHODS

Sample collection and preparation

Adult specimens (n = 9) of the pen shell *A.* (*S.*) *maura* (approximately 30 cm) were collected from La Paz Bay, Baja California Sur, Mexico, through the fishing cooperative Organización de Pescadores Rescatando la Ensenada (OPRE). Viscera, comprising all soft tissues except the adductor muscle (including the digestive gland, gonads, mantle, gills, and associated internal organs) (n = 3 per batch; 150 g each), were dissected under sterile conditions, frozen at -20°C, and stored for one week until further processing.

Protein extraction

Three batches of viscera (n = 3 per batch; 150 g each) were homogenized in 150 mM citric acid-sodium citrate buffer (pH 5.6) using a commercial blender, reaching a final volume of 500 mL. Homogenates were filtered through sterile gauze, centrifuged at 12,000 *g* for 20 min at 4°C (Thermo Scientific, Sorvall RC 6+), and the supernatants were collected and stored at -20°C.

Enzymatic hydrolysis

Protein extracts (100 mL), containing 2,264 mg of soluble protein, were hydrolyzed using Wobenzym (Société des Produits Nestlé S.A., Vevey, Switzerland), a commercial multi-enzyme preparation containing pancreatin, bromelain, papain, lipase, amylase, trypsin, and chymotrypsin. Hydrolysis was performed using 10 g of powdered Wobenzym. According to the manufacturer, each 850 mg contains bromelain (112,500 U.I.), papain (12,000 U.I.), trypsin (60,000 U.N.F.), and chymotrypsin (1,000 U.N.F.). Based on the amount used in this study, the total proteolytic activity applied during hydrolysis corresponded to approximately 1.32×10^6 U of bromelain, 1.41×10^5 U of papain, 7.06×10^5 U of trypsin, and 1.18×10^4 U of chymotrypsin. Hydrolysis was carried out at 30°C under constant agitation at 100 rpm for 24 h. This reaction time was selected to promote progressive proteolysis of the viscera protein extract under mild conditions and to generate peptide fractions with reduced molecular weights, including intermediate fractions of 20-29 kDa and shorter peptide sequences that may be associated with antimicrobial activity. Subsequently, the enzymatic hydrolysates were centrifuged at 12,000 × *g* for 20 min at 4°C using a Beckman J2-HS. Enzymatic activity was then terminated by heating at 90°C for 10 min. The resulting supernatant was collected and filtered through sterile Whatman 542 filter paper (VWR International) to remove lipids and non-hydrolyzed material. Finally, all samples were aliquoted under sterile conditions and stored at -20°C until further physicochemical and biological analyses.

Protein quantification

Protein quantification of the visceral and hydrolyzed extracts, as well as the chromatographic fractions, was performed using the method described by Lowry et al. (1951), using bovine serum albumin (BSA) as a standard. Absorbances were recorded at 750 nm with a BIORAD iMark™ microplate reader. Measurements were performed in triplicate.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein profiles (50 µg) from crude extracts, hydrolysates, and fractions were analyzed by SDS-PAGE (Laemmli 1970) using a Mini-PROTEAN® II Cell system (Bio-Rad, 2000, CA, USA). Samples were mixed with 4× sample buffer containing 50 mM Tris-HCl, pH 6.8, 20% glycerol, 10% SDS, 10% β-mercaptoethanol, and 0.05% bromophenol blue. Then, the samples were boiled for 10 min and loaded onto a 10 or 12% polyacrylamide gel. A broad-range molecular weight standard (Bio-Rad, 1610317, CA, USA) was also loaded into the gel. Electrophoresis was performed at 90 V and room temperature using a vertical electrophoresis unit (Bio-Rad Protean II, CA, USA). After electrophoresis, the gels were stained for 2 h with Coomassie blue solution, containing Coomassie Brilliant Blue R-250 (0.05% w/v), 7% v/v acetic acid, and 40% v/v methanol. Protein bands were then visualized by soaking the gel in a destaining solution (7% v/v acetic acid and 40% v/v methanol).

Gel filtration chromatography

Proteins obtained by enzymatic hydrolysis were fractionated by high-resolution gel filtration chromatography using a Sephacryl S-100 column (Cytiva Lifesciences) with dimensions of 1.6×35 cm. The column was pre-equilibrated with 150 mM citric-citrate sodium buffer (pH 5.6). Protein fractions were eluted from the column using the same buffer at a flow rate of 1.13 mL min^{-1}. A total of eighty fractions, each with a volume of 1.5 mL, were collected. Protein peak fractions were determined based on their absorbance at 280 nm. All samples were preserved at -4°C until further use.

Antimicrobial activity

Antimicrobial activity was first assessed using a radial diffusion method in agar, following the protocols described by Valgas et al. (2007) and Fothergill (2012). Strains used were *Enterobacter aerogenes*, *Salmonella enteritidis*, *S. typhimurium*, *Shigella flexneri* (ATCC 12022), and *S. sonnei* (ATCC 9290). Petri dishes containing LB agar were inoculated with 100 µL of bacteria (1×10^8 cells mL^{-1}). Afterward, paper filter disks (Whatman, ¼, VWR) were placed in the petri dishes, and 10 µL of visceral extract or a chromatographic protein fraction was applied to each disk. The agar plates were then incubated at 37°C for 24 h. Antimicrobial activity was determined by measuring inhibition zone diameters via digital image analysis on the ChemiDoc system (Bio-Rad Laboratories, Hercules, CA, USA) equipped with the integrated antibiogram analysis module. Measurements were automatically calculated in millimeters using the software's internal calibration parameters and pixel-to-distance conversion. In parallel, a vernier caliper was used to identify and manually verify the inhibition zones preliminarily. Gentamicin (50 µg mL^{-1}) served as a positive control; 150 mM citric-citrate sodium buffer (pH 5.6) served as a negative control.

Dynamic growth inhibition assay in liquid culture

The dynamic growth inhibition assay in liquid culture was performed according to the bacterial growth kinetics modeling approach described by Zwietering et al. (1990), using optical density monitoring under controlled incubation conditions. Briefly, an early stationary-phase inoculum was prepared by incubating single bacterial colonies overnight (12 h) at 37°C in LB Broth Base (Sigma-Aldrich, St. Louis, MO, USA). Overnight cultures were diluted in fresh LB medium to an OD600 of 0.12. Inoculum (100 µL per well) was added to a sterile 96-well microplate. Peptide fractions or control solutions (both positive and negative) were then added to final protein concentrations of 12.5, 25, 100, and 200 µg mL^{-1}, respectively. Growth was monitored by measuring OD600 every 60 min in aerobic conditions at 37°C with interval shaking in a Thermo Scientific™ Scan system. Growth inhibition was assessed by calculating the area under the curve (AUC) from the OD600 readings, and inhibitory effects were determined by comparing the AUC of treated samples with that of untreated controls. All the experiments were performed in triplicate.

Statistical analysis

All experiments were conducted in triplicate unless otherwise indicated. Results were expressed as means ± standard deviation (SD). Statistical analyses, including comparisons of AUC values, were performed using RStudio 2025.09.1 (RStudio, PBC, Boston, MA, USA).

RESULTS

Protein quantification and electrophoretic analysis

Crude protein extracts from *A.* (*S.*) *maura* viscera showed a protein concentration of 22.64 ± 2.85 mg mL^{-1} across three biological replicates, corresponding to 2,264 mg of soluble protein in 100 mL of extract. After hydrolysis, the protein concentration increased significantly to 33.63 ± 8.16 mg mL^{-1} (Table 1), representing a 1.48-fold increase. This enhancement in protein concentration reflects effective proteolytic activity by Wobenzym, likely due to the breakdown of large proteins into smaller peptide fragments. Electrophoretic profiles (SDS-PAGE) confirmed the hydrolysis process: crude extracts displayed multiple high-molecular-weight bands (Fig. 1), whereas hydrolysates exhibited a predominance of low-molecular-weight peptides (<29 and ~24 kDa) (Fig. 2).

Protein fractionation by gel filtration

Size-exclusion chromatography of the hydrolysates revealed two major peaks, indicating the presence of distinct peptide populations across the three replicates of *A.* (*S.*) *maura* viscera hydrolysates (Fig. 2a). The chromatographic profiles showed consistent peak patterns among the replicates. However, a slight offset was observed between them. Notably, the first hydrolysate showed lower absorbance at the initial peak than the second and third hydrolysates, which showed greater similarity in peak intensity and shape.

The first peak, eluting between fractions 10 and 20, was consistently observed across all three hydrolysates and corresponded to a mixture of low-molecular-weight peptides. The second peak, between fractions 30 and 40, corresponded to proteins or peptides of higher molecular weight. These interpretations were further supported by SDS-PAGE analysis (Fig. 2b), which revealed a common banding pattern in selected fractions associated with both peaks. Specifically, fractions 10-20 displayed bands below ~21 kDa, while fractions 30-40 contained a predominant band around ~29 kDa (Fig. 2b), confirming the molecular separation suggested by the chromatographic profiles.

Quantification of protein content in each collected fraction, using the Lowry method, showed a distribution pattern consistent with the chromatographic absorbance at 280 nm. Protein concentrations within the first peak (fractions 10-20) ranged from 24.8 to 456.875 µg mL^{-1}. In contrast, the second peak (fractions 30-40) showed significantly higher concentrations, ranging from 2,421.875 µg mL^{-1} to a maximum of 4,562.5 µg mL^{-1}, particularly pronounced in the second and third hydrolysates. These findings confirm the effective enzymatic hydrolysis of viscera proteins and the distinct separation of peptide populations by molecular weight through gel filtration chromatography.

Antimicrobial activity assays: disk diffusion assay

The diameter of the inhibition zones was measured by digital image analysis using the ChemiDoc system (Bio-Rad Laboratories, Hercules, CA, USA) equipped with the integrated antibiogram analysis module. In parallel, a vernier caliper was used to identify and verify inhibition zones preliminarily. During the primary screening, the disk diffusion assay showed that several peptide fractions inhibited the tested bacterial strains, as evidenced by the formation of measurable inhibition zones around the treated disks. The degree of antimicrobial activity varied across both fractions and

Table 1. Protein concentration of crude extract, viscera, and hydrolysates from *A.* (*Servatrina*) *maura.*

Sample	Volume (mL)	Protein concentration (mg mL^{-1})	Total protein (mg)
Crude extract	145 ± 7.07	22.64 ± 2.85	3303.61 ± 559.77
Hydrolysates	138.33 ± 6.23	33.63 ± 8.16	4701.52 ± 1331.42

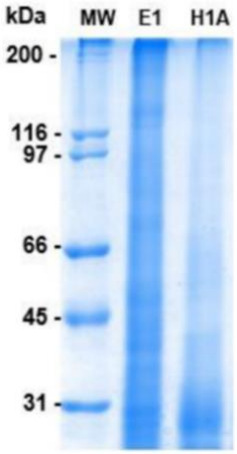

Figure 1. SDS-PAGE of unhydrolyzed and hydrolyzed viscera from *A.* (*S.*) *maura*. 10% SDS-PAGE of unhydrolyzed (E1) and hydrolyzed viscera (H1A). MW: molecular weight marker.

bacterial species. Among them, fractions 24, 25, and 39 consistently demonstrated the strongest inhibitory effects against *E. aerogenes*, *S. enteritidis*, *S. typhimurium*, *S. flexneri*, and *S. sonnei*. The inhibition zone diameters for these fractions ranged from 6.5 to 10 mm, which were comparable to, or slightly lower than, those of the gentamicin control discs (Fig. 3).

Further evaluation of chromatographic fractions 10-40 showed that multiple fractions exhibited antimicrobial activity across all three hydrolysate batches. As summarized in Table 2, the second hydrolysate, derived from *A.* (*S.*) *maura* viscera, produced a greater number of active fractions compared to the first and third batches. This hydrolysate was particularly effective against *S. sonnei*, and several fractions were active against multiple bacterial strains.

A notable trend was observed among low-molecular-weight fractions (<21 kDa), which exhibited stronger antimicrobial activity. Specifically, fraction 39 consistently showed broad-spectrum inhibitory effects across multiple strains. Reassessment of the active fractions confirmed the effectiveness of the positive control, which inhibited all tested strains. In contrast, the negative control produced results comparable to those of the less active fractions, with inhibition zones not exceeding 6.5 mm. Fractions in the 20-25 range demonstrated the most consistent and pronounced inhibitory activity, particularly against *S. enteritidis*, *S. flexneri*, and *S. sonnei*.

Dynamic growth inhibition assay

The dynamic growth inhibition assay in liquid culture was performed according to the bacterial growth kinetics modeling approach described by Zwietering et al. (1990), using optical density monitoring under controlled incubation conditions. The growth-inhibitory effects of selected peptide fractions-fraction 1 (a pool composed of peptides with molecular weights <21 kDa), fraction 2 (peptides <29 kDa), and fraction 3 (29 kDa)- were dynamically quantified by calculating the AUC over a 24-h incubation period (Zwietering et al. 1990). For testing, hydrolysates from batches H1, H2, and H3 were combined and pooled into each respective fraction. Each pooled fraction consisted of three hydrolysates, each originating from a different batch, and was subsequently used in the bioassays.

All tested fractions resulted in a measurable reduction in bacterial growth compared with untreated controls. Among them, fractions 2 and 3 demonstrated the most pronounced inhibitory effects across all four bacterial strains tested, *E. aerogenes*, *S. flexneri*, *S. typhimurium*, and *S. sonnei* (Fig. 4). Fraction 3 showed strong activity, reducing bacterial growth by approximately 40% at lower concentrations, with inhibition reaching up to 80% at the highest concentration tested (200 µg mL^{-1}). This effect was especially notable against *S. flexneri* and *S. typhimurium*.

Conversely, fraction 2 exhibited a biphasic effect. While it inhibited growth at higher concentrations, *S. flexneri* and *S. sonnei* showed increased growth at lower concentrations (around 12.5 µg mL^{-1}), suggesting a possible hormetic or adaptive fitness response. A similar stimulatory effect was observed with fraction 1 in *S. sonnei*, even at the lowest concentration tested.

Interestingly, *E. aerogenes* was consistently inhibited by all fractions, indicating broad-spectrum sensitivity. In contrast, the variable responses observed in *S. flexneri* and *S. sonnei*, particularly under low concentration exposure

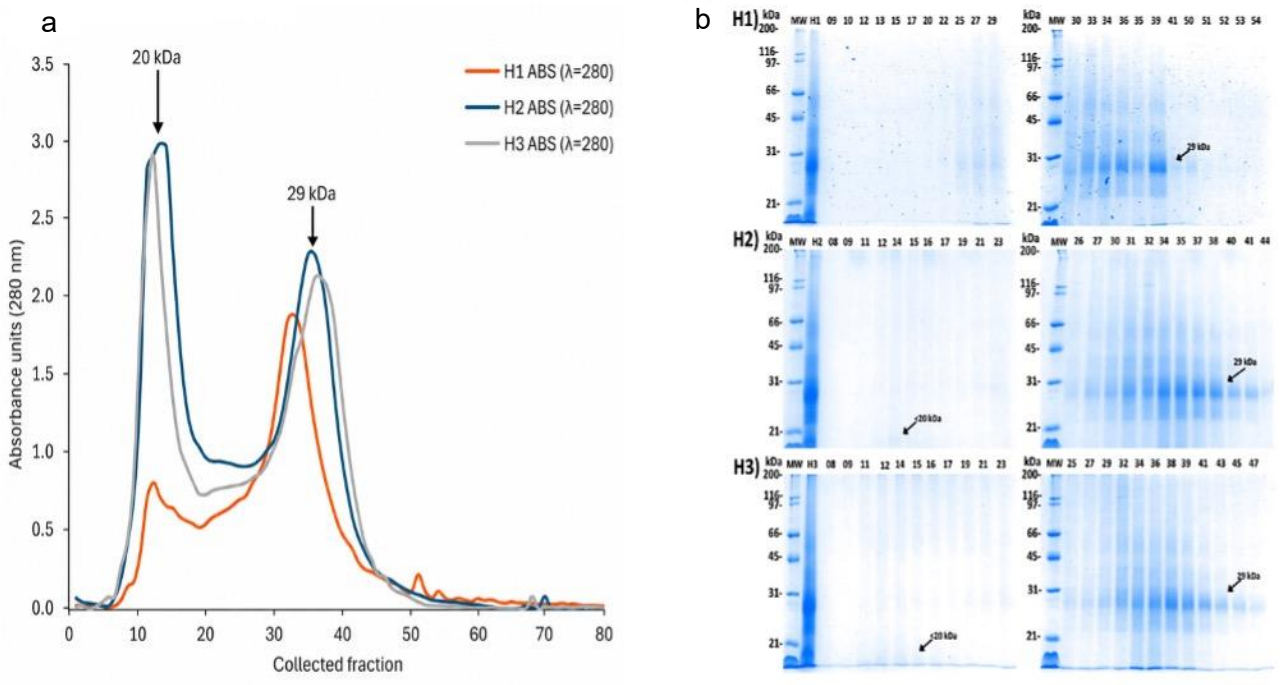

Figure 2. Chromatographic fractionation and electrophoretic characterization of *Atrina* (*Servatrina*) *maura* viscera hydrolysates. a) Size-exclusion chromatographic profiles of three hydrolysate batches obtained from *A.* (*S.*) *maura* viscera: H1, H2, and H3. Protein/peptide fractions were eluted with 150 mM sodium citrate-citric acid buffer at pH 5.6. Peaks represent the elution profile of each hydrolysate batch, and numbered fractions correspond to the collected eluates. b) Corresponding SDS-PAGE profiles of selected eluted fractions from each hydrolysate batch using 12% polyacrylamide gels: batch 1, H1; batch 2, H2; and batch 3, H3. MW indicates the broad-range molecular weight marker. Fraction numbers in the gels correspond to those shown in the chromatographic profiles. Electrophoretic analysis corroborated the molecular weight distribution of the eluted fractions, with early fractions showing predominantly low-molecular-weight peptide bands and later fractions, particularly fractions 30-40, showing higher-molecular-weight components of approximately 29 kDa.

to fractions 1 and 2, point to complex, possibly strain-specific adaptation or signaling responses.

DISCUSSION

Bioactive peptides exert diverse biological activities that are strongly influenced by their amino acid composition, net charge, hydrophobicity, amphipathicity, and molecular size. *In vitro* hydrolysis of marine protein sources has demonstrated that controlled proteolysis can generate bioactive peptides with activities comparable to, or even greater than, those of their naturally occurring counterparts (Ngayen et al. 2012). Several strategies have been developed to obtain bioactive peptides *in vitro*, including thermal treatment, acid hydrolysis, enzymatic digestion, and, more recently, subcritical water extraction (Hao et al. 2019). Among these approaches, enzymatic hydrolysis provides greater control over the degree of hydrolysis and peptide size distribution, enabling the targeted generation of peptide fractions with defined molecular characteristics (Hou et al. 2007). Although this method may involve higher operational costs, it offers greater reproducibility and selectivity than non-specific chemical treatments.

Food-grade proteolytic preparations have been extensively used to produce functional protein hydrolysates (García-Moreno et al. 2017, Neves et al. 2017, Sukkhown et al. 2018). However, variations in enzyme specificity, catalytic efficiency, and cleavage patterns can significantly influence the molecular weight distribution and amino acid composition of the released peptides (Tavano 2013). In the present study, enzymatic hydrolysis of *A.* (*S.*) *maura* visceral proteins using Wobenzym generated peptide-rich fractions with distinct molecular weight distributions below 29 kDa. The increase in soluble protein concentration after hydrolysis, together with SDS-PAGE profiles showing

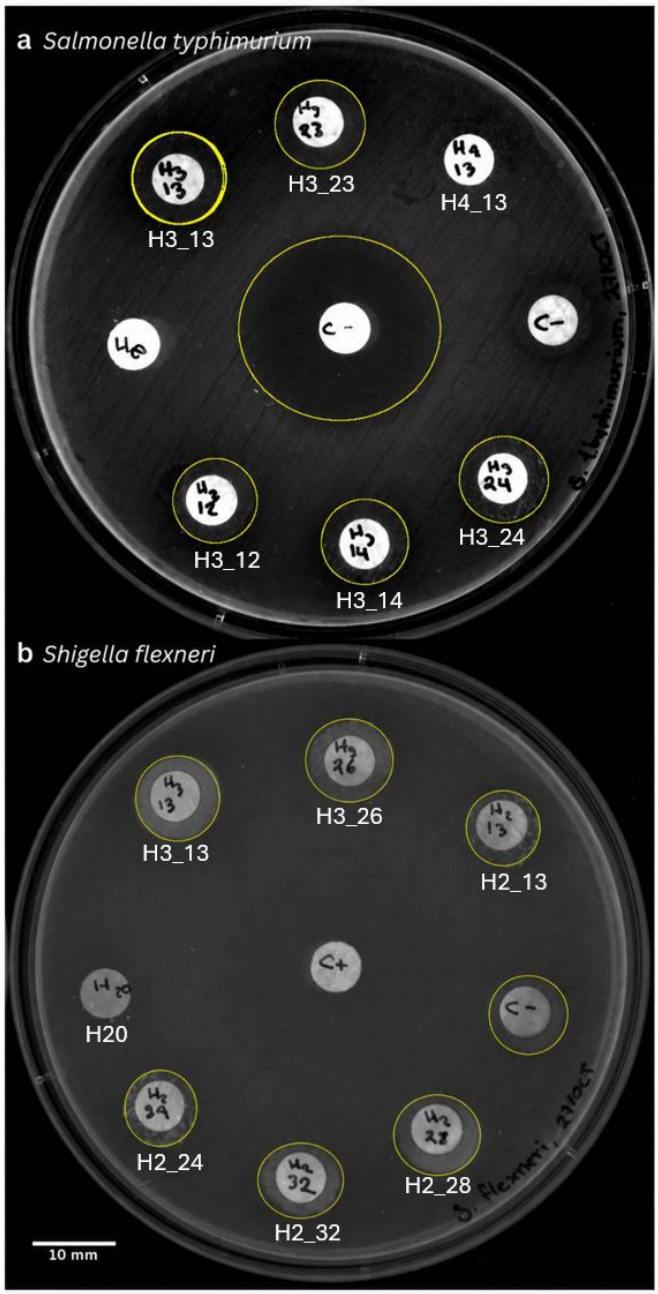

Figure 3. Representative images of inhibition zones produced by peptide fractions from *Atrina* (*Servatrina*) *maura* viscera hydrolysates against *Salmonella typhimurium* and *Shigella flexneri* using agar diffusion assays. a) *S. typhimurium* and b) *S. flexneri.* Yellow circles indicate the inhibition zones selected for diameter measurement. C+ represents the positive antimicrobial control, and C- represents the negative control. The labels on the discs indicate the hydrolysate number and fraction number: H: hydrolysate; F: fraction. For example, H3_13 corresponds to hydrolysate 3, fraction 13. Inhibition was quantified as the diameter of the clear zone surrounding each disc and reported in millimeters. Scale bar = 10 mm.

a shift toward lower-molecular-weight bands, confirmed the effective proteolytic cleavage of precursor macromolecules.

Size-exclusion chromatography further resolved two principal peptide populations: fractions enriched in peptides below approximately 21 kDa and fractions containing peptides around 29 kDa. In *A.* (*S.*) *maura*, fractions enriched in lower molecular weight peptides exhibited stronger inhibitory effects, particularly in dynamic growth assays against *E. aerogenes*, *S. flexneri*,

Table 2. Antibacterial activity of gel filtration fractions from enzymatic hydrolysates of *A.* (*Servatrina*) *maura* viscera.

Bacterial strain	Active fractions (hydrolysate replicates)
Enterobacter aerogenes	25, 35, 39
Salmonella enteritidis	24, 25, 26, 28, 35, 36, 39
Salmonella typhimurium	13, 28, 32, 35, 39
Shigella flexneri	13, 20, 21, 23, 25, 24, 28, 32, 39
Shigella sonnei	13, 17, 23, 26, 30, 39

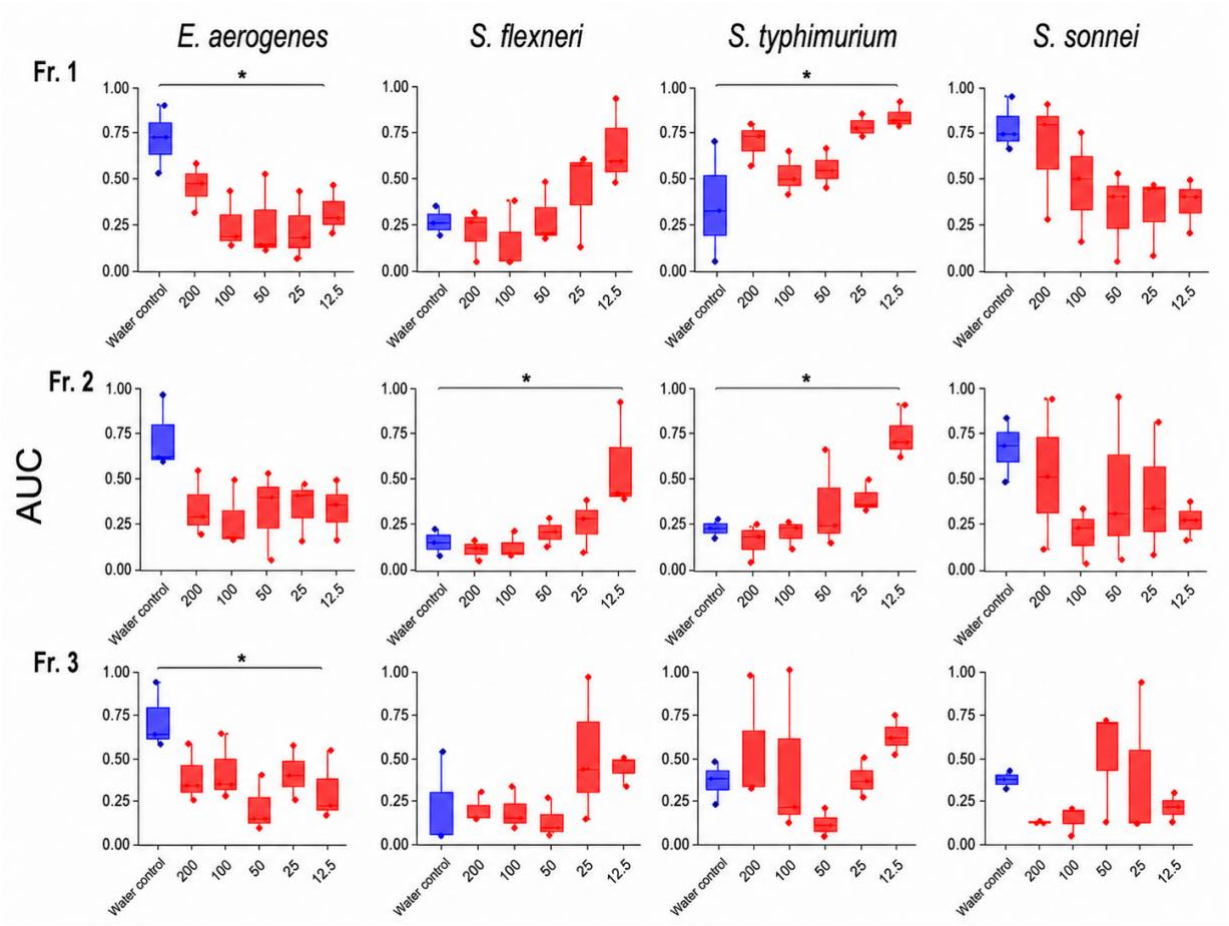

Figure 4. Dynamic inhibition of bacterial growth by peptide fractions obtained from *Atrina* (*Servatrina*) *maura* viscera. Bacterial strains, including *Enterobacter aerogenes*, *Shigella flexneri*, *Salmonella typhimurium*, and *Shigella sonnei*, were exposed to chromatographic peptide fractions (12.5-200 µg mL^{-1}) obtained from *A.* (*S.*) maura viscera. Growth inhibition was evaluated over a 24-h incubation period at 37°C by calculating the area under the bacterial growth curve (AUC). AUC values were normalized to the untreated water control; lower normalized AUC values indicate stronger inhibition of bacterial growth. Data are presented as boxplots showing the distribution of normalized AUC values for water control and peptide-fraction treatments. Water control is shown in blue, whereas peptide treatments are shown in red. Statistical differences between each treatment and the water control were assessed after testing homogeneity of variance using Levene's test. When homoscedasticity was met, one-way ANOVA followed by Tukey's *post-hoc* test was applied; when homoscedasticity was not met, Kruskal-Wallis followed by Dunn's *post-hoc* test was used. Asterisks indicate significant differences compared with the water control ($P < 0.05$). Experiments were performed in triplicate. Fr.: fraction.

S. typhimurium, and *S. sonnei*. This observation is consistent with previous reports indicating that antimicrobial efficacy is frequently associated with reduced peptide size, which may facilitate diffusion through the outer membrane of Gram-negative bacteria and promote interactions with negatively charged lipopolysaccharides (Li & Yu 2015). Smaller peptides often exhibit enhanced membrane permeability and faster bactericidal kinetics due to their ability to adopt amphipathic conformations that destabilize lipid bilayers (Cheung et al. 2015, Zasloff 2019).

The antimicrobial activity observed in the *A.* (*S.*) *maura*-derived peptide fractions are consistent with previous reports on mollusk-derived extracts and hydrolysates. Low-molecular-weight peptides from *Anodonta cygnea* hydrolysates have shown inhibitory activity against *Escherichia coli*, *Salmonella typhi*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Listeria monocytogenes* (Heydari et al. 2024). Similarly, solvent extracts from the Korean pen shell *Atrina pectinata* have demonstrated inhibitory activity against *E. coli*, *Pseudomonas aeruginosa*, *Enterobacter sakazakii*, and *Salmonella typhimurium* (Suyeon et al. 2011). Antimicrobial activity has also been reported for extracts or hydrolysates from other mollusks, including *Placuna placenta* against *Bacillus cereus* (Salido et al. 2022), *Haliotis fulgens* against *Proteus mirabilis*, *P. aeruginosa*, *S. typhimurium*, *S. typhi*, *E. aerogenes*, *E. coli*, *S. aureus*, and *B. subtilis*, and *Haliotis corrugata* against *P. mirabilis* (Rivera-Perez et al. 2023). Together, these findings support the relevance of mollusks as sources of peptide fractions with antimicrobial potential.

The growth inhibition observed against *Salmonella* and *Shigella* species may reflect the presence of bioactive peptide sequences released from *A.* (*S.*) maura viscera during enzymatic hydrolysis and subsequently enriched through chromatographic fractionation. These peptides may share functional features with innate immune effector molecules naturally involved in mollusk host defense. Marine invertebrates are continuously exposed to complex microbial communities and rely predominantly on innate immune mechanisms to respond to microbial pressure. Therefore, their peptide-based defense strategies represent a valuable source of antimicrobial molecules with potential biotechnological applications (Guryanova & Ovchinnikova 2022).

The inhibitory effect observed against *Salmonella* and *Shigella* species may also be related to the specific physiological features of these Gram-negative enteric pathogens. Both genera possess an outer membrane enriched in lipopolysaccharides, which acts as a permeability barrier against antibiotics and host defense molecules, but also represents an initial interaction site for cationic and amphipathic antimicrobial peptides. Therefore, inhibiting these Gram-negative pathogens is particularly relevant, as their outer membranes serve as a selective barrier that often limits the efficacy of conventional antimicrobial agents.

In *Salmonella*, membrane remodeling systems such as PhoPQ/PmrAB can modify LPS structure and confer resistance to cationic antimicrobial peptides, highlighting the importance of the bacterial envelope in peptide susceptibility. In addition, *Salmonella* possesses stress-response mechanisms that allow survival under acidic, osmotic, oxidative, and intracellular conditions, which are closely associated with virulence and antimicrobial tolerance. In contrast, *Shigella* is characterized by a very low infectious dose, acid resistance, epithelial cell invasion, intracellular motility, and toxin-mediated disruption of host cells. These traits make *Shigella* highly adapted to the intestinal environment and may influence its susceptibility to membrane-active compounds. Thus, the antimicrobial activity of *A.* (*S.*) *maura*-derived peptide fractions against these pathogens may be explained, at least in part, by their ability to interact with the negatively charged Gram-negative envelope, destabilize LPS-rich membranes, increase permeability, and promote leakage of intracellular components (Hassanin et al. 2025).

Although classical antimicrobial peptides typically range from 2 to 10 kDa, the activity observed in fractions approaching approximately 29 kDa suggests that antimicrobial functionality may not be restricted exclusively to short peptides. These higher-molecular-weight fractions may contain partially hydrolyzed proteins or peptide aggregates that retain exposed cationic or amphipathic domains generated during enzymatic processing, thereby enabling interactions with bacterial membranes. Similar observations have been reported in marine-derived hydrolysates, where bioactivity may arise from larger fragments containing functional antimicrobial motifs (Palacios-Abrantes et al. 2018, Rivera-Perez et al. 2023, Davoudi et al. 2024). indicating that the exposure of active regions, rather than molecular size alone, may contribute to antimicrobial performance.

Although membrane permeabilization was not directly assessed in this study, the inhibitory effect observed for the *A.* (*S.*) *maura*-derived peptide fractions are consistent with membrane-targeting mechanisms commonly described for antimicrobial peptides. In

Gram-negative bacteria, the outer membrane contains negatively charged lipopolysaccharides, which may favor the initial electrostatic interaction with cationic and amphipathic peptides. This interaction may promote peptide accumulation at the bacterial surface, followed by outer membrane destabilization and increased permeability. Once the peptides reach the cytoplasmic membrane, they may induce membrane deformation, lipid disorganization, pore-like lesions, and leakage of intracellular molecules, ultimately leading to bacterial growth inhibition or cell death (Roncevic et al. 2022).

The concentration-dependent inhibition observed in dynamic growth assays further supports the possible involvement of membrane-associated effects. The pronounced reduction in bacterial growth, reaching up to 80% inhibition at 200 µg mL^{-1}, demonstrates that these peptide fractions substantially affected bacterial proliferation. Differential responses among strains, particularly the variability observed in *Shigella flexneri* and *S. sonnei* at lower concentrations, may reflect differences in outer membrane composition, efflux systems, or stress-response pathways, as previously described for Gram-negative bacteria exposed to cationic peptides (Nam et al. 2015, Chen et al. 2024b). The biphasic responses detected at subinhibitory concentrations may be interpreted as a hormetic-like response, in which low peptide concentrations impose moderate membrane or metabolic stress, transiently activating bacterial adaptive mechanisms. In contrast, higher concentrations exceed the bacterial compensatory capacity, resulting in marked growth inhibition. In this sense, subinhibitory exposure to marine-derived peptide fractions could stimulate bacterial stress responses, including membrane remodeling, efflux pump activation, changes in envelope composition, or metabolic adjustments that temporarily favor survival. This phenomenon is particularly relevant for functional food or pharmaceutical applications because suboptimal peptide concentrations may fail to inhibit target pathogens fully and could promote transient stress adaptation. Therefore, defining effective inhibitory concentrations, dose-response behavior, peptide stability, and activity under application-specific conditions is essential before considering these fractions for applied use.

The antimicrobial potential of *A.* (*S.*) *maura*-derived peptide fractions are also relevant in the broader context of marine antimicrobial peptide discovery. The emergence of multidrug-resistant pathogens has intensified the search for alternatives to conventional antibiotics. Marine organisms have emerged as valuable reservoirs of antimicrobial peptides because of their high biological diversity and constant exposure to complex microbial communities. These peptides are widely distributed across several marine phyla, including Porifera, Cnidaria, Annelida, Arthropoda, Mollusca, Echinodermata, and Chordata, where they function as key components of innate immune defense. In this regard, mollusks are especially relevant because they rely predominantly on innate immune mechanisms to respond to microbial pressure in aquatic environments. Marine antimicrobial peptides are commonly characterized by broad-spectrum activity, distinctive mechanisms of action, relatively low cytotoxicity, and promising stability, which make them attractive molecules for biotechnological and therapeutic applications (Thomas & Antony 2023). Therefore, the antimicrobial effect observed in the *A.* (*S.*) *maura*-derived peptide fractions are consistent with the recognized potential of marine invertebrates as sources of bioactive peptides and support the valorization of mollusk-derived tissues and by-products as sources of antimicrobial compounds.

The relationship between molecular size distribution and antimicrobial activity observed in this study further suggests that structural features play an important role in determining peptide bioactivity. Enzymatic hydrolysis may have contributed not only to increased soluble protein recovery but also to the generation of peptide populations with different molecular sizes and functional properties, which supports the idea that controlled proteolysis can be used as a strategy to release bioactive sequences from marine proteins and modulate their biological activity. In this context, antimicrobial peptides may be generated from larger precursor proteins via hydrolytic processes, yielding low-molecular-weight fractions enriched in active sequences.

This concept has been reported in other biological systems. For example, Albergaria et al. (2010) showed that *Saccharomyces cerevisiae* CCMI 885 secretes proteinaceous compounds smaller than 10 kDa, mainly in the 2-10 kDa fraction, that inhibit the growth of non-*Saccharomyces* yeasts. Similarly, Santos et al. (2022) reported that peptide extracts smaller than 10 kDa obtained through *S. cerevisiae* autolysis contained antibacterial peptide candidates encrypted within stress-related and metabolic proteins. These peptides, identified by nano-LC-MS/MS, were associated with abundant proteins involved in stress response and cellular metabolism and were generated through induced proteolysis and proteasomal activity. These findings support the hypothesis that hydrolysis and

fractionation of *A.* (*S.*) *maura* visceral proteins may have released and concentrated low-molecular-weight peptides with antimicrobial potential. Such peptides could originate from structural, metabolic, or stress-related precursor proteins naturally present in the viscera and may contribute to the inhibition observed against *Salmonella* and *Shigella* species.

The activity observed against *Salmonella* and *Shigella* species is especially relevant given their clinical and environmental significance (WHO 2017, Anderson et al. 2023). Gram-negative bacteria possess a complex outer membrane barrier that limits the penetration of many conventional antibiotics, making them suitable models for evaluating membrane-active biomacromolecules. Overall, these results suggest that *A. maura* visceral hydrolysates contain peptide populations with relevant antimicrobial potential against Gram-negative enteric pathogens. However, the active sequences and their precise mechanisms remain elucidated. Future studies integrating LC-MS/MS-based peptide identification, physicochemical profiling, membrane permeabilization assays, cytotoxicity evaluation, and synergy analysis will be essential for validating their potential as marine-derived antimicrobial candidates.

CONCLUSIONS

This study demonstrates that peptide fractions derived from *A.* (*S.*) *maura* viscera possess significant antimicrobial activity against clinically relevant Gram-negative bacteria, including multidrug-resistant strains of *Salmonella* and *Shigella*. Enzymatic hydrolysis and chromatographic fractionation enabled the identification of low-molecular-weight peptides that inhibit bacterial growth in both solid and liquid media. These findings highlight the potential of *A.* (*S.*) *maura* viscera as a sustainable source of novel antimicrobial agents and lay the groundwork for future research focused on peptide characterization, mechanism of action elucidation, and in vivo applications in therapeutic and aquaculture settings.

Credit author contribution

H.S. Almazán-Pérez: methodology, and formal analysis; N.Y. Hernández-Saavedra: conceptualization and funding acquisition; V. Juárez-Arellano: methodology, formal analysis and writing-original draft; C. Rivera-Pérez: conceptualization, funding acquisition, validation, methodology, formal analysis, writing-original draft, supervision, review and editing. All authors have read and accepted the published version of the manuscript.

Conflict of interests

The authors declare no competing interests.

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