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# Purification and characterization of four antibacterial peptides from protamex hydrolysate of Atlantic mackerel (*Scomber scombrus*) by-products

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ABSTRACT

Proteins from fish by-product sources are valuable source of bioactive peptides and show promise as functional foods ingredients. The objective of the present study was to isolate and characterize antibacterial peptides from protamex hydrolysates of Atlantic mackerel (*Scomber scombrus*) by-products. Four sequences SIFIQRFTT (P4), RKSGDPLGR (P8.1), AKPGDGAGSGPR (P8.2) and GLPGPLGPAGPK (P11) were identified in peptide fractions separated using RP-HPLC. At 200 μg $mL^{-1}$, while peptides P8.1, P8.2 and P11 exhibited partial inhibition, P4 totally inhibited tested Gram-positive (*Listeria innocua*) and Gram-negative (*Escherichia coli*) bacterial strains. These results suggest that the protein hydrolysate derived from mackerel by-products could be used as an antimicrobial ingredient in both functional food and nutraceutical applications.



## 1. Introduction

Fish processing wastes are considered to be a safe, high-protein material with several nutritional benefits, and a good pattern of essential amino acids. Additionally, proteins from fish sources show promise as functional foods ingredients as they are valuable source of bioactive peptides encrypted within primary amino acid sequences and released upon enzymatic hydrolysis [1]. These bioactive molecules are short peptides with only 3–20 amino acid residues. In recent years, an increasing numbers of bioactive peptides were identified in protein hydrolysates from fish processing materials and shown to possess antioxidant [2–4], anticoagulant [5], antihypertensive [6,7] and antimicrobial [8–10] properties.

Antimicrobial peptides (AMPs) from marine organism hydrolysates are increasingly isolated and reported during the last few years. Liu et al. [8] first identified an AMP (named CgPep33) in oyster muscle proteolysed using a combination of alcalase and bromelin. The CgPep33 is cysteine-rich peptide composed by seven residues [C, L, E, D, F, Y, I and G], and inhibits growth of several Gram-positive and Gram-negative bacteria and fungi. More recently, different antibacterial peptides from barbel muscle protein hydrolysates digested with alcalase were identified and shown inhibitory against several pathogenic bacteria [11,12]. Similarly, antibacterial peptides fractions isolated from proteolysed snow crab and Atlantic rock crab by-products were shown inhibitory toward several Gram-positive and Gram-negative bacteria [13,14]. Antibacterial peptide fraction was also isolated from the pepsin hydrolysate of half-fin anchovy [10]. A total of five cationic peptides and three anionic peptides were identified. Similarly, a decapeptide (GLSRLFTALK) inhibiting *Staphylococcus aureus* was identified from anchovy cooking waste water by-product [15].

Previously, we have shown that Atlantic mackerel represents a valuable source of food with high nutritional value [16]. Mackerel hydrolysate using protamex was shown to contain high amounts of proteins and well-balanced fat and amino acid composition [16]. Recently, we isolated antibacterial peptide fractions in mackerel processing by-products hydrolysed using commercial enzymes (protamex, papain, neutrase, and flavourzyme) [17]. These active fractions had low molecular weights and were active against Gram-positive (*Listeria innocua*) and Gram-negative (*Escherichia coli*) bacteria. The objective of the present study was to identify and to characterize AMPs from mackerel by-products hydrolysed using protamex.

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## 2. Materials and methods

### 2.1. Materials

Atlantic mackerel (*Scomber scombrus)* was provided by the Aquaculture and Fisheries Innovation Center (Merinov, Gaspé, Canada). The fishes were caught in the Atlantic Ocean by trawling in the Magdalen Islands. After the capture, the specimens were immediately placed on ice and transferred to the laboratory where they were eviscerated. The coproducts were mixed and homogenized for 3 min and then vacuum wrapped and frozen at −20 °C. The coproducts included all the organs usually found in the abdomen: viscera, digestive gland, stomach gonads, heart, intestines, liver and spleen. The enzyme protamex (EC 3.4.21.62; Novozymes A/S, Bagsvaard, Denmark) was used for hydrolysis of Mackerel coproducts.

### 2.2. Microorganisms and culture conditions

Microbial growth inhibition assays were performed using Gram-positive (*L. innocua* HPB 13) and Gram-negative (*E. coli* MC4100) bacterial strains. Both strains were grown aerobically in Tryptic Soy Broth with 0.6% yeast extract (w/v) (TSB; Difco, Sparks, MD, USA). *L. innocua* were incubated at 30 °C and *E. coli* were incubated at 37 °C.

### 2.3. Hydrolysis of proteins

Mackerel coproducts were hydrolysed using protamex as previously described in Ennaas et al. [17]. Briefly, 200 g of coproducts samples were thawed overnight at 4 °C and homogenised with an equal amount of water (w/v). Hydrolysis conditions (pH 8, 50 °Cand300 rpm) were monitored for 24 h with pH adjustment using 5N NaOH. The hydrolysis was stopped by heating the mixtures at 90 °C for 10 min. Insoluble and non-hydrolysed materials were centrifuged at 20 000 × *g* for 30 min at 4 °C and recovered soluble fractions were freeze-dried and stored at −20 °C until subsequent use.

### 2.4. Determination of antibacterial activity

Antibacterial activity was assayed using a microtest polystyrene microplate method (96-well Microtest, Becton Dickinson Lab-ware, Sparks, MD, USA) as previously described [18]. Briefly, microplates were loaded with two fold serial dilutions of peptide samples in tryptic soy broth and seeded with approximately $1 \cdot 10^4$ cfu per well using log-phase culture of the indicator strain diluted in tryptic soy broth to $0.5–1.0 \cdot 10^6$ cfu $mL^{-1}$. The suspension brought the final volume in each well to 175 μL. The microplates were then incubated with indicator strain for 24 h and absorbance at 595 nm was measured every 20 min using an Infinite F200 PRO photometer (Tecan US Inc., Durham, NC, USA). Microbial counts were determined in each well after 24 h.

### 2.5. Purification of peptides

Hydrolysate of mackerel coproducts was precipitated using acetone (v/v) and centrifuged for 20 min at 8000 × *g* at 4 °C. The resulting supernatant was separated using sep-pack C18 with 3 solutions of acetonitrile (ACN) (10, 60 and 100%), and collected fractions were evaluated for their antibacterial activity. Peptides from active fraction were separated using a Gold HPLC system (Beckman–Coulter, Brea, CA, USA) with an analytical reversed-phase C18 column (150 × 4.6 mm; Phenomenex, Torrance, CA, USA). Linear gradients were conducted from 2% to 60% solvent B (0.1% TFA in ACN) over solvent A (0.1% TFA) in 75 min with a flow rate of 1 mL $min^{-1}$. Elution was monitored by UV absorbance at 214 nm. Concentrations of protein in the fractions were determined by the bicinchoninic acid (μBCA) method (BCA, Pierce Biotechnology, Rockford, IL, USA).

### 2.6. Identification of peptides by MALDI-TOF/TOF

Matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry (MALDI-TOF) was performed on a AB SCIEX 4800 Plus MALDI-TOF/TOF instrument using alpha-cyano-4-hydroxycinnamic acid as matrix. The spectra were acquired using the 4000 Series Explorer Software (AbSciex, v 3.2.3). The PEAKS Studio software (Bioinformatics Solutions, v.5.3) was used for spectra analysis and DENOVO sequencing.

## 3. Results

### 3.1. Protein hydrolysis, purification and antibacterial activity of peptides fractions

Mackerel coproducts were hydrolysed using protamex for 24 h. The biochemical composition of the resulting supernatant was characterized and was identical to our previous report [17]. The soluble fraction contained high peptide content and exhibited antibacterial activity, as previously shown. This active fraction was then fractionated on sepak cartridge C18 and eluted with different acetonitrile gradient 0, 10, 60 and 100%. The most active fraction (60%) was loaded to RP-column C18 and HPLC profile monitored at 214 nm, as shown in Fig. 1. A total of thirteen fractions were collected and protein concentration ranged from 0.218 to 2 mg $mL^{-1}$. All fractions were evaluated for their inhibitory activity against *L. innocua* HPB13, as shown in Fig. 2. At concentration of 200 μg $mL^{-1}$, the peptide fractions 4 and 11 totally inhibited growth of *L. innocua* HPB13. In a lesser extent, P1, P2, P3, P6, P7, P8, P10, P12, and P13 induced a partial inhibition of listerial growth. Comparative growth inhibition of peaks P4, P6, P8 and P11 against *E. coli* MC4100 and *L. innocua* HPB13 are presented in Fig. 3. The peak 4 (at 200 μg $mL^{-1}$) exhibited a total inhibition of both bacterial strains. While *L. innocua* was totally inhibited in presence of peak 11, the latter has inhibited only the three-quarter of *E. coli* cells. The peaks 6 and 8 exhibited a high percentage of inhibition corresponding to 31.7 and 46.8% of *E. coli* MC4100 and 19.5 and 28.8% against *L. innocua* HPB13.

### 3.2. Identification of antibacterial peptides

The peptides present in active fractions were loaded onto MALDI-TOF/TOF and their sequences presented in Table 1. A total of four sequences SIFIQRFTT (P4), RKSGDPLGR (P8.1), AKPGDGAGSGPR (P8.2) and GLPGPLGPAGPK (P11) were identified in peptide fractions 4, 8 and 11, respectively. All peptides had a lower mass than 1200 Da and have a positive charge at neutral pH with isoelectric points below 10 and were composed mainly of aliphatic amino acids. The identification of the peptides in fraction 6 was not possible because of weak quantities recovered. As example, Fig. 4 illustrates MS and MS/MS identification of peptide P4, a 1094.7 Da nonapeptide containing a dehydrated threonine at position 8. The peptide is a cationic (+1) and possesses a high ratio of hydrophobic residues (44%). The peptide P4 was identified as fragment f(291–302) in protein (PETMA-S4RP006) from fish *Petromyzon marinus* (sea lamprey). Similarly, the peptide P11 was found f(291–302) in fish collagen protein 0S4R4C5 PETMA. Prediction of peptide secondary structure indicated that P8.1, P8.2 and

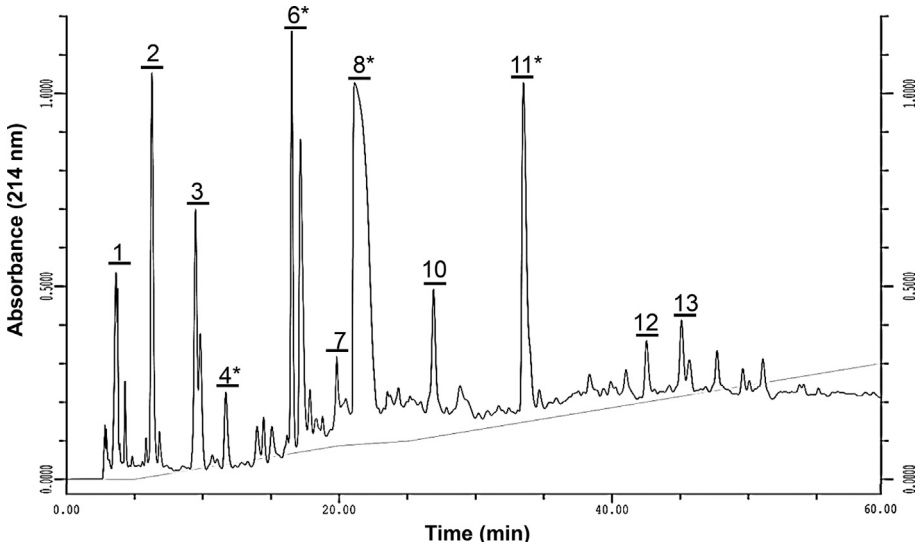


**Fig. 1.** Reversed-phase HPLC profile of active fraction recovered from mackerel by-products hydrolysed for 24 h using protamex. Most potent peaks are asterisk marked.

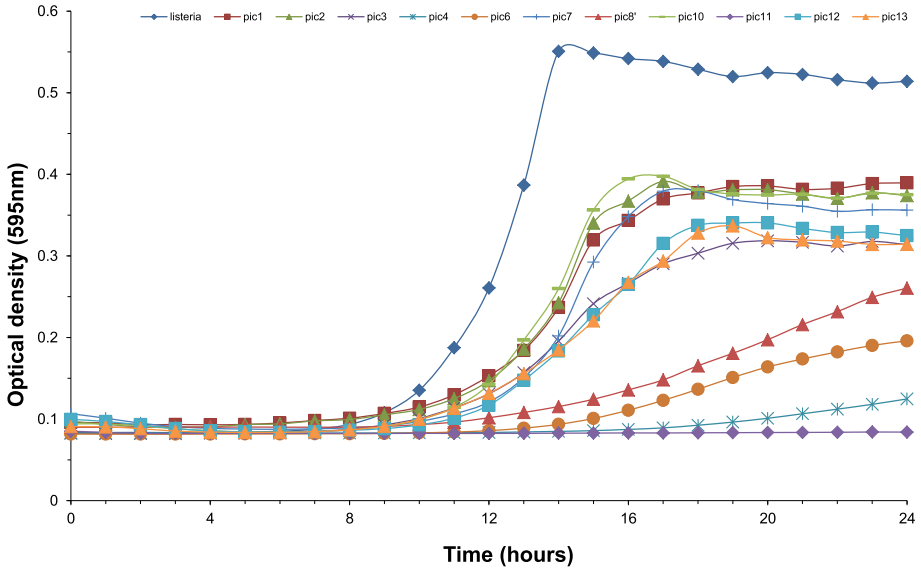


**Fig. 2.** Determination of antimicrobial activity of the collected HPLC fractions recovered from mackerel by-product hydrolysate against *L. innocua* HPB13 at 200 μg $mL^{-1}$.

P1 should adopt random coil structures, whereas peptide P4 could form and extended strand and coil structures.

## 4. Discussions

Fish proteins are a valuable source of bioactive peptides encrypted within primary amino acid sequences and released upon enzymatic hydrolysis during food processing or transit throw gastrointestinal tract. During last decade, an increasing number of bioactive peptides were identified in protein hydrolysates from fish processing wastes and shown to possess antioxidant [2–4,19], antihypertensive [6,7], antitumor [20], anticoagulant [5], or antimicrobial [8–10] properties. Previously, we reported on antibacterial peptide fractions in mackerel processing coproducts hydrolysed using commercial enzymes (protamex, papain, neutrase, and flavourzyme) [17]. These active fractions had low molecular weights and were active against Gram-positive (*L. innocua*) and Gram-negative (*E. coli*) strains. In this study, protamex hydrolysates of mackerel coproducts was fractionated and antibacterial peptides presents were identified and characterized.

Four sequences SIFIQRFTT (P4), RKSGDPLGR (P8.1), AKPGDGAGSGPR (P8.2) and GLPGPLGPAGPK (P11) were identified in peptide fractions separated using RP-HPLC. A total inhibition was obtained with peptide 4 for both Gram-positive and Gram negative bacterial strains. No homology were observed for P8.1 and P8.2 with other AMPs in APD and CAMP databases. Extended comparison with known AMPs revealed homology of peptide P4 with peptide MiAMP1 [21]. First five residues of P4 (SIFIQ) are localised at the C-terminal of MiAMP and could be implicated in the peptide action. Similarly, the PLGPAG sequence of peptide P11 has been detected on antimicrobial sequences of bacteriocin ABP-118α and P1-Hp-1971, isolated from bacteria (*Lactobacillus salivarius* UCC118) and skin secretions of frog (*Hypsiboas pulchellus*), respectively [22,23]. P11 is a proline rich peptide, a common characteristic of several AMPs reported in literature. The sequence of P11 was identified from collagen hydrolysate. Previously, collagen has also been

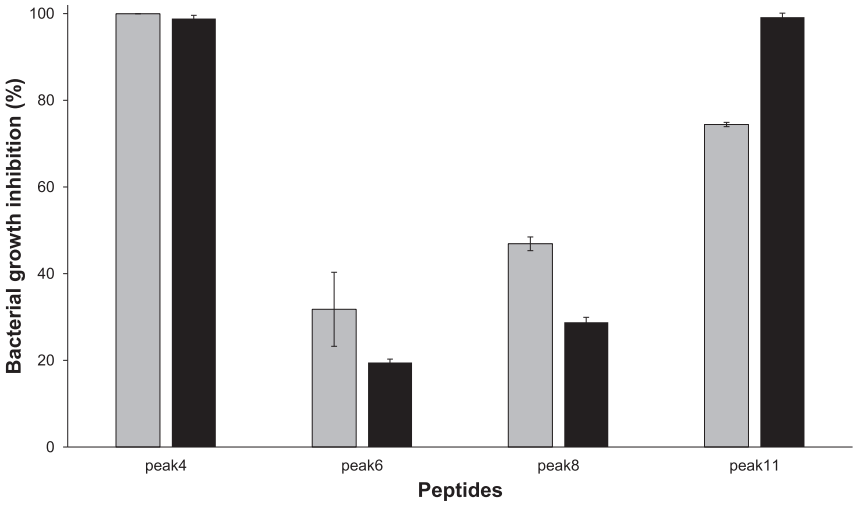


**Fig. 3.** Bacterial growth inhibition of L. innocua HPB13 (gray) and *E. coli* MC4100 (black) in presence of peptide fractions 4, 6, 8 and 11 individually at 200 μg $mL^{-1}$.

reported to exert bactericidal and fungicidal properties when applied as a coating to wool-based materials [24].

AMPs from fish hydrolysates are increasingly isolated and reported during the last few years. In instance, Liu et al. [8] have isolated CgPep33 from oyster (*Crassostrea gigas*) muscle hydrolysed using a combination of alcalase and bromelin. CgPep33 is a cysteine-rich AMP composed by seven residues [C, L, E, D, F, Y, I and G]. The peptide inhibited the growth of the Gram-positive (*Bacillus subtilis* and *S. aureus*) and negative bacteria (*Pseudomonas aeruginosa* and *E. coli*) and fungi (*Botrytis cinerea* and *Penicillium expansum*). Similarly, antibacterial peptides fractions were reported from proteolysed snow crab (*Chionoecetes opilio*) and Atlantic rock crab (*Cancer irroratus*) by-products with activity against Gram-negative and Gram-positive bacteria [13,14]. In another study, Song et al. [10] isolated and characterized the peptide fraction from the pepsin hydrolysate of half-fin anchovy (*Setipinna taty*). A total of five cationic peptides (MLTTPPHAKYVLQW, SHAATKAPPKNGNY, PTAGVANALQHA, QLGTHSAQPVPF and VNVDERWRKL) and three anionic peptides (LATVSVGAVELCY, NPEFLASGDHLDNLQ and PEVVYECLHW) were identified. More recently, an AMP with sequence GLSRLFTALK was identified from anchovy cooking wastewater by-product and was inhibitory against *S. aureus* [15]. Different antibacterial peptides from barbel (*Barbus callensis*) muscle protein hydrolysates digested with alcalase were identified and shown inhibitory against several pathogenic bacteria [11,12].

Additionally, fish secrete several AMPs involved in host defense mechanisms, reviewed in Refs. [25,26]. During the past few years, a number of AMPs have been identified from fish skins. In instance, Su [27] have isolated from the skin mucus of yellow catfish an AMP, referred as pelteobagrin (GKLNLFLSRLEILKLFVGAL). The peptide exhibited a large activity spectrum against a wide range of bacteria without hemolytic activity. Similarly, an antimicrobial protein (41 kDa) generated using a trypsin digest was identified from the skin of *Epinephelus fario* and was shown active against gram-negative and gram-positive bacteria [28]. Another study reported on three AMPs (named TH2–2, TH2–3, and TH1–5) from tilapia

**Table 1**
Amino acid sequence (based on MALDI-TOF/TOF spectra) and corresponding fragment of antibacterial peptides found in reserved-phase HPLC fractions after 24 h of hydrolysis by protamex.

| Fraction | Sequence | Observed/Theoretical mass | pI | Net charge | Hydrophobic ratio (%)[a] | Ions assigned to peptide |
|---|---|---|---|---|---|---|
| 4 | SIFIQRFTT<br>ceeeeeccc | 1094.7/1112.19 | 9.47 | +1 | **44.4** | **a4**: 433.281;**b3**: 348.192;**b4**: 461.276;**b4H2O**: 70.0287;<br>**y1**: 120.0655; **y3**: 350.171; **y4**: 506.2722; **y5**: 634.33.07;<br>**y6**: 747.4148; **y4NH3**: 489.246; **y5NH3**: 617.304;<br>**y6NH3**: 730.388; **ya**: 214.155; 276.182; 359.219; 600.362;<br>**yb**:285.167; 231.113; 242.15; 432.235 |
| 6 | ND | ND | – | – | – | – |
| 8.1 | RKSGDGPLGR<br>ccccccccc | 1042.5/1042.16 | 10.84 | +2 | **10.0** | **y1**: 175.1299; **y4**: 442.2370; **y5**: 499.2682; **y6**: 614.2960;<br>**y4NH3**: 425.2144; **y5NH3**: 482.2406; **b4**: 396.1614; **b5**: 511.2065;<br>**b6**: 568.2476; **b7**: 639.2954; **b8**: 696.3491;**y1**: 175.1355;<br>**y3**: 329.1948; **y4**:416.2381; **y5**: 473.2682; **y6**: 544.3010;<br>**y7**: 601.3380; **y8**: 716.3679;**y10**: 870.4507;**y4NH3**: 399.2142 |
| 8.2 | A**K**PGDGAGSGPR<br>**ccccccccccc** | 1111.6/1069.19 | 8.79 | 0 | **16.6** | **y6NH3**: 527.2759; **y7NH3**: 584.375;**y8NH3**: 699.3702 |
| 11 | GLPGPLGPAGP**K**<br>ccccccccccc | 1102.5/1060.26 | 8.75 | 0 | **25.0** | **b3**: 268.1656; **b4**: 325.187; **b5**: 422.2398; **b6**: 535.3239;<br>**b7**: 592.3453; **b8**: 689.3981; **b9**:760.4352; **y5**: 511.2875;<br>**y7**: 681.393; **y8**: 778.4458; **y9**: 835.4672; **y10NH3**: 915.4934;<br>**ya**: 127.087;<br>**yb**: 155.082; 252.1343; 268.166; 325.187; 422.24; 535.324 |

PTM: dehydrated (−18) amino acids are underlined; acetylated (+42) amino acids are bolded.
The secondary structures (c: coil, e: extended strand) of the identified peptides were predicted by the protein sequence analysis server (Network Protein Sequence@nalysis) at https://npsa-prabi.ibcp.fr/.
[a] Calculated as percentage of hydrophobic residues (Ile, Val, Leu, Phe, Cys, Met, Ala, and Trp) in the sequence.

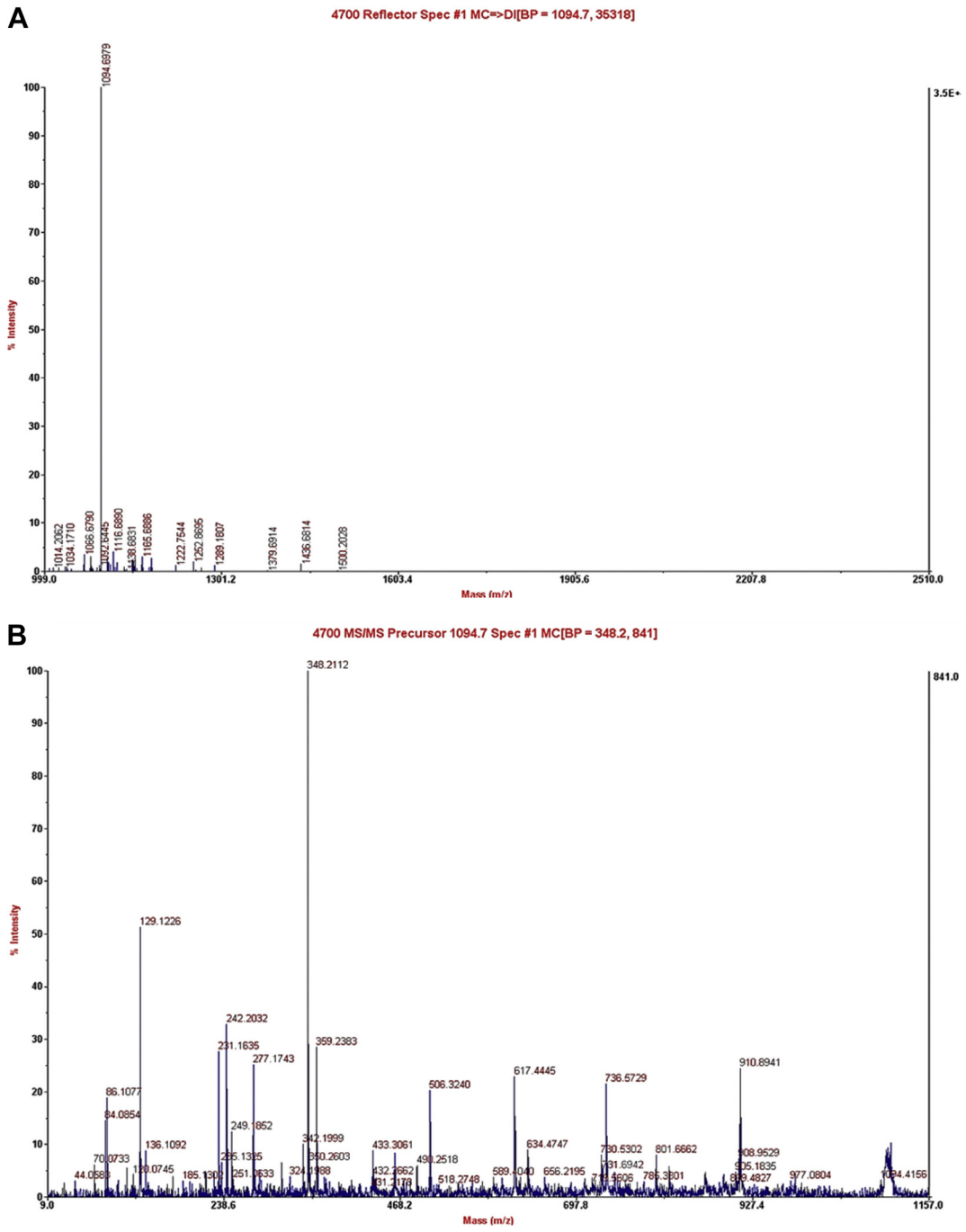


**Fig. 4.** Identification of peptide P4 sequence and ions corresponding to the mass determined by MALDI-TOF/TOF. (A) MS spectrum of peptide P4; (B) MS/MS spectrums relevant to the species at m/z 1094.69 observed in P4.

with similarity to Japanese flounder (*Paralichthys olivaceus*) and sea bream (*Chrysophrys major*) hepcidins [29]. The Hepcidin TH1–5 presented also antitumor activity against tumor cell lines. Synthetic TH1–5 inhibited the proliferation of tumor cells and reduced colony formation in a soft agar assay. Initially described as bactericidal molecules, AMPs are now considered to be multifunctional molecules.

Extended comparison revealed that sequence of P11 was highly similar (7/9 identical) to peptide PGPLGLTGP, an ACE inhibitor that was previously isolated from skate skin hydrolysate [30]. Moreover, Banerjee and Shanthi [31] have reported on isolation of novel bioactive peptides (D1 and E2) hydrolyzed by bacterial collagenase from bovine Achilles tendon collagen having ACE-inhibitory properties. The peptide D1 was the most potent inhibitor. Fragments f(15–26) and f(3–14) of identified peptides D1 and E2 share 58.3 and 50.0% of similarity with the peptide P11, respectively. Previously, an overlapping sequence GFHypGP hydrolyzed by fermentation (using *Aspergillus oryzae*) of collagen of porcine skin was shown to exert antihypertensive effect in spontaneously hypertensive rats [32]. Thus, besides of its antibacterial action, P11 could be probably antihypertensive. Several potent ACE inhibitory hydrolysates and peptides have been isolated from collagenous materials, not only from marine sources such as fish skins and cartilage, scales, squid tunics and sea cucumbers, but also from

porcine and bovine skin gelatin, chicken legs and leg bone, reviewed in Ref. [33]. During the last years, several studies have been focused on the production of bioactive peptides from collagen by-product sources using different enzymes. These collagenous materials from the fish processing industries include skins, bones, tunics and scales. While the antioxidant and antihypertensive/ACE inhibitor activity of collagen and gelatin-derived peptides is considered the most documented, numerous other bioactivities were reported for these molecules, including antimicrobial activity, immunomodulatory activity, lipid-lowering effect, mineral binding capacity and beneficial effects on skin, bone or joint health, reviewed in Ref. [33]. Due to the incidence of mad cow disease, food industry was less attracted by collagen hydrolysate peptides from pig or bovine by-products. Alternatively, marine processing waste sources such as skin collagen gained more attention last years, and collagen remains a very important protein in food industry as a source of bioactive peptides.

Bioactive peptides derived from fish processing by-products have potential in the development of functional foods. An increasing number of bioactive peptides with antimicrobial properties from fish by-products origin have been identified, which has spurred new directions in food safety and human health. Hence, fish-derived AMPs may have great potential for use as active ingredients in functional foods and food supplements.

## Conflict of interest

None.

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## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.091.

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