Evidence of Antibacterial Activities in Peptide Fractions Originating from Snow Crab (*Chionoecetes opilio*) By-Products

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Abstract Antibacterial peptide fractions generated *via* proteolytic processing of snow crab by-products exhibited activity against Gram-negative and Gram-positive bacteria. Among the bacterial strains tested, peptide fractions demonstrated inhibitory activity against the Gram-negative bacteria such as *Aeromonas caviae*, *Aeromonas hydrophila*, *Campylobacter jejuni, Listonella anguillarum*, *Morganella morganii*, *Shewanella putrefasciens*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* and against a few Gram-positive bacteria such as *Listeria monocytogenes*, *Staphylococcus epidermidis* and *Streptococcus agalactiae*. The principal bioactive peptide fraction was comprised mainly of proteins and minerals (74.3 and 15.5%, respectively). Lipids were not detected. The amino acid content revealed that arginine (4.6%), glutamic acid (5.3%) and tyrosine (4.8%) residues were represented in the highest composition in the antibacterial peptide fraction. The optimal inhibitory activity was observed at alkaline pH. The *V. vulnificus* strain, most sensitive to the peptide fraction, was used to develop purification methods. The most promising chromatography resins selected for purification, in order to isolate peptides of interest and to carry out their detailed biochemical characterization, were the SP-Sepharose^{TM} Fast Flow cation exchanger and the Phenyl Sepharose^{TM} High Performance hydrophobic interaction media. The partially purified antibacterial peptide fraction was analyzed for minimum inhibitory concentration (MIC) determination, and the value obtained was 25 μg ml^{-1}. Following mass spectrometry analysis, the active peptide fraction seems to be a complex of molecules comprised of several amino acids and other organic compounds. In addition, copper was the main metal found in the active peptide fraction. Results indicate the production of antibacterial molecules from crustacean by-products that support further applications for high-value bioproducts in several areas such as food and health.

Keywords Antibacterial activities · Peptide fractions · Snow crab by-products

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Introduction

Antimicrobial peptides (AMPs) are known as important components of the innate immune system in a variety of organisms including both vertebrates and invertebrates [6, 22]. Generally, AMPs contain 15–45 amino acid residues [7] and possess cationic and amphipathic properties, which allow for interactions with the membrane of cells in order to neutralize and kill pathogenic microorganisms [28, 31, 32]. Although there are extensive studies on AMPs, only a few

have been isolated and completely characterized from crustaceans [17, 24, 37] even though these organisms, living in a microbe-rich oceanic environment, are capable of producing AMPs as a first-line defense system [30, 33].

Schnapp et al. [35] reported in 1996 the isolation and the partial sequencing of a proline-rich 6.5-kDa antibacterial peptide from the shore crab *Carcinus maenas* while Relf et al. [33] isolated a cysteine-rich 11.5-kDa polypeptide from the same crab species in 1999. Another characterization was the penaeidin family of AMPs from the Pacific white shrimp *Litopenaeus vannamei* [11]. Thus, using the molecular approach of expressed sequence tag (EST) analysis, putative homologues of the 11.5-kDa polypeptide (from *C. maenas*) and the penaeidins have been characterized in other shrimp species, including *L. setiferus* [2, 15], *Penaeus monodon* [38] and *P. japonicus* [34]. Callinectin, a cationic 3.7-kDa peptide, was isolated and partially characterized from the blue crab, *Callinectes sapidus* [23]. Additionally, some peptide fragments generated from the C-terminal part of crustacean hemocyanin have been shown to possess antibacterial activities such as the cationic 1.9-kDa peptide named astacidin 1 [24]. Haug et al. [17] detected high antibacterial activity against several bacterial strains in haemocyte extracts of the spider crab, *Hyas araneus*, and further isolated and purified AMPs from these extracts [17, 37]. Also, Herbinière et al. [19] reported the isolation and the characterization of armadillin, a novel glycine-rich antibacterial peptide from the woodlouse *Armadillidium vulgare* (terrestrial isopod, crustacean). More recently, the isolation and characterization of two AMPs from the American lobster, *Homarus americanus*, were reported [3]. In most of the crustacean species studied so far, AMPs have shown cationic properties and have been located in the hemolymph and/or in the hemocytes [18]. Interestingly, antifungal peptides were generated from the C-terminus of shrimp hemocyanin which presented a negative net charge [12]. To our knowledge, only one other novel anionic antibacterial peptide, named scygonadin, was recently isolated from the seminal plasma of the mud crab, *Scylla serrata* [41].

The increase in antibiotic-resistant pathogenic bacteria has stimulated the search for alternative antibacterial agents from natural sources [18, 27]. Many antibacterial peptides show a high specificity for prokaryotes and a low toxicity for eukaryotic cells [36, 42]. Their mechanism of action is still largely unknown, although several seem to act by forming pores in the cell membrane and making susceptible bacteria leaky [13] allowing for many interesting activities (antibiotic, fungicidal, virucidal and tumoricidal). Based on these facts, such peptides are being developed for use as a novel class of antimicrobial agents, which could be extended to various food, biomedical and health applications [28].

Even though AMPs were isolated from other crustaceans, here we report, for the first time, the presence of AMPs originating from hydrolysates of snow crab by-products. In previous work published from our laboratory, it was mentioned that many bioactive compounds fractionated from snow crab by-products should be further characterized for their potential applications [4]. Hence, the detection of antibacterial activity in enzymatic hydrolyzed snow crab by-products fractions was performed using reference and environmental bacterial strains of interest in the food and health sectors. The most sensitive bacterial strain was used to pursue the development of more specific purification methods. The method developed was used for the purification of peptides of interest and to carry out their detailed biochemical characterization (molecular weight, amino acid composition). Determination of mineral elements and characterization of organic compounds, in the active partially purified fraction, were performed by several analyses using mass spectrometry.

Materials and Methods

Enzymatic Hydrolyzed Fractions of Snow Crab By-Products

Snow crab hydrolysate fractions were produced at the Aquatic Products Technology Centre (CTPA, MAPAQ, Gaspé, QC, Canada) according to the procedure described previously [4]. Briefly, 100 kg of water was added to 100 kg of grinded snow crab by-products (cephalothorax shells, digestive systems including hepatopancreas and hemolymph), the total volume was heated to 40°C, and 100 g of the proteolytic enzymes blend Protamex® was added to start the hydrolysis. After 60-min hydrolysis at 40°C, the temperature in the tank was gradually increased to a temperature of 85°C to inactivate the proteases before the liquid content was decanted and run through a two-phase separator to isolate insoluble fraction from the aqueous fraction. The aqueous fraction was centrifuged in order to separate lipids from proteins. The protein solution was then passed through multiple filtration steps to separate the proteins by molecular weight: 50-kDa retentate (>50 kDa), 1-kDa retentate (50–1 kDa), nano-filtration retentate (1 kDa–200 Da) and reversed osmosis retentate (<200 Da). Final fractions were kept frozen until analyses (−20°C).

Bacterial Strains and Growth Media

Reference strains used in this study are listed in Table 1. All strains were maintained in nutrient media with 10%

Table 1 Bacterial strains tested and their sensitivity to a low molecular weight peptide fraction obtained from snow crab by-products

Organism	Strain	Culture conditions	Sensitivity to peptide fraction
Aeromonas caviae	ATCC 15468	MH, 22°	+
Aeromonas hydrophila	LSPQ 3308	TSA, 25°	+
Campylobacter jejuni	LEA 79	MH, 37°	+
Enterobacter aerogenes	ATCC 13048	MH, 35°	−
Enterobacter sakazakii	ATCC 51329	MH, 35°	−
Escherichia coli	ATCC 25922	MH, 35°	−
Listeria monocytogenes	ATCC 19112	TSA, 30°	+
Listeria monocytogenes	ATCC 19114	TSA, 30°	+
Listeria monocytogenes	ATCC 19115	TSA, 30°	−
Listeria monocytogenes	ATCC 35152	TSA, 30°	−
Listonella anguillarum	ATCC 19264	MH, NaCl 1.5%,35°	+
Morganella morganii	ATCC 25830	MH, 35°	+
Pseudomonas aeruginosa	ATCC 27853	MH, 35°	−
Pseudomonas aeruginosa	LEA GN36	MH, 35°	−
Salmonella typhimurium	ATCC 14028	MH, 35°	−
Salmonella typhimurium	LEA 625	MH, 35°	−
Shewanella putrefasciens	LSPQ 2227	MH, 25°	+
Staphylococcus aureus	ATCC 25923	MH, 35°	−
Staphylococcus aureus	LEA 56	MH, 35°	−
Staphylococcus epidermidis	Clinic	MH, 35°	+
Streptococcus agalactiae	ATCC 19615	MH, 35°	+
Vibrio parahaemolyticus	ATCC 17802	TSA,NaCl 1.5%,35°	+
Vibrio parahaemolyticus	LEA GN39	TSA,NaCl 1.5%,35°	−
Vibrio vulnificus	LSPQ 2967	TSA,NaCl 1.5%,35°	+
Vibrio vulnificus	LEA GN38	TSA,NaCl 1.5%,35°	+

ATCC American type culture collection, *LEA* laboratoire d'expertise et d'analyses alimentaires, *MAPAQ* Québec, *LSPQ* laboratoire de santé publique du Québec *MH* Mueller–Hinton (BD-Difco), *TSA* Tryptic Soy Agar (BD-Difco)

+ Presence of an inhibition zone in the agar diffusion assay

− Absence of an inhibition zone in the agar diffusion assay

glycerol at −80°C. Before the experiments, strains were sub-cultured at least two times, in their respective media.

Determination of the Inhibitory Spectrum

The resulting protein hydrolysate fractions, crude and concentrated (fivefold) by evaporation using a Titan vapor trap (Jouan, Winchester, USA), were evaluated for antibacterial activity against 24 bacterial strains on the basis of a slightly modified agar well diffusion assay described by Himelbloom et al. [20]. Briefly, each overnight culture was diluted 1:10 and 2 ml used to seed 200 ml of the selected culture media containing 0.75% (w/v) agar, corresponding to approximately 5×10^5 cells/ml. The plates were prepared by pouring the inoculated media and allowed to solidify, and 6-mm wells were punched. Aliquots (80 μl) of the peptide samples were added to the wells. The plates were incubated at 4°C for 2 h to permit diffusion into the solid media and thereafter at the appropriate temperature for 18 h. Clear zones around the wells indicated antibacterial activity. One bioactive peptide fraction (nano-filtration retentate; 1 kDa–200 Da) was selected for further analyses, based on this antibacterial activity.

Bioactive Peptide Fraction Analyses

Chemical Composition Determination

Initial bioactive peptide fraction (nano-filtration retentate; 1 kDa–200 Da) was characterized to determine its chemical composition as described previously [4]. Moisture and minerals (ash) were measured using the official methods of analysis of the Association of Official Analytical Chemists (No. 950.46 and No. 938.08) [1]. Lipids were analyzed using a method based on the Bligh and Dyer method [6]. Crude proteins were determined by the Kjeldahl method (nitrogen × 6.25) adapted from the official method of A.O.A.C. No. 988.05 [1].

Amino Acid Analyses

Two different methods were used for amino acid determination of the initial bioactive peptide fraction as described previously [4]. The EZ: faast amino acid analysis procedure was used for tryptophan determination requiring an alkaline hydrolysis. The EZ: faast amino acid analysis procedure (Phenomenex, Torrance, CA, USA) was used

and consisted of a solid-phase extraction step followed by derivatization and liquid/liquid extraction. Derivatized samples were analyzed by gas chromatography with flame ionization detection (FID). The AccQ-Tag amino acid analysis procedure (Waters, Mississauga, ON, Canada) was used for the determination of amino acids resisting to acidic hydrolysis (including taurine). The AccQ-Tag method is a pre-column derivatization technique for peptide and protein hydrolysate amino acids. The amino acids were separated by reversed-phase high-performance liquid chromatography (HPLC) and quantified by fluorescence detection.

pH Studies

In order to measure peptide fractions (nano-filtration retentate; 1 kDa–200 Da) activity as a function of pH, fractions concentrated (fivefold) using a Titan vapor trap (Jouan, Winchester, USA), at a starting pH of 7.7, were adjusted with 2 N NaOH or 1 N HCl at pH 5, 6, 7, 8 and 9. Antibacterial activity was evaluated for each fraction using agar diffusion test (80 µl) as described previously. The bacterial strain *Vibrio vulnificus* LEA GN38 was used as sensitive strain.

Purification Process

The initial developmental work was performed using 1-ml resin columns. Several ion exchange chromatography (IEX) and hydrophobic interaction chromatography (HIC) resins were tested using HiTrap™ IEX Selection Kit and HiTrap™ HIC Selection Kit (GE Healthcare, Baie d'Urfé, QC, Canada). Bioactive peptide purification at a larger scale was achieved using 20-ml resin XK16/20 columns (GE Healthcare, Baie d'Urfé, QC, Canada) in order to generate more bioactive peptide material. Following each purification step, the antibacterial activity was followed by agar well diffusion assay and by size exclusion chromatography against *V. vulnificus* LEA GN38 . The scale-up of the purification was performed as follows:

Ion Exchange Chromatography

Bioactive fraction from the snow crab processing (580 ml), previously filtered (1.2-µm Glass microfibre filters, 70 mm, Whatman, VWR Canlab, Mississauga, ON, Canada; 0.45-µm HAWP nitrocellulose membrane filters, Millipore, Bedford, MA, USA) and adjusted at pH 9 with 2 N NaOH, was captured using a SP-Sepharose™ Fast Flow cation exchange column (GE Healthcare, Baie d'Urfé, QC, Canada). The column (GE Healthcare, Baie d'Urfé, QC, Canada) was connected to a FPLC system (AKTÄ Explorer 100, GE Healthcare, Baie d'Urfé, QC, Canada). The system was equipped with UV

(215, 254, 280 nm), conductivity and pH monitors, which were connected to an autosampler and a fraction collector. The chromatograms were obtained and analyzed using the UNI-CORN 5.0 software. The column was equilibrated with 10 CV (CV = column volume) of 50 mM bicine buffer at pH 9 (buffer A). The peptide solution was applied to the column at a rate of 156 cm/h; then, the column was washed successively with buffer A alone and buffer A containing 1 M NaCl. Bioactive peptide fractions were eluted from the column with buffer A.

Hydrophobic Interaction Chromatography

The active fractions collected from the SP-Sepharose column were pooled (1,046 ml), and salt was added in order to obtain a concentration of 1 M NaCl. This solution was then applied to a Phenyl Sepharose™ High Performance hydrophobic interaction column (GE Healthcare, Baie d'Urfé, QC, Canada) connected to the same FPLC system (AKTÄ Explorer 100, GE Healthcare, Baie d'Urfé, QC, Canada). The column was equilibrated with buffer A containing 1 M NaCl. The peptide solution was applied to the hydrophobic interaction chromatography (HIC) column at the rate of 156 cm/h; the column was washed with the same equilibration buffer, followed by a second wash with buffer A. Bioactive peptide fractions were eluted with buffer A. The active fractions were pooled (275 ml) and concentrated (fivefold) using a Titan vapor trap (Jouan, Winchester, VI, USA). The material was stored at 4°C.

Analytical Methods

Total Protein Assay

Protein concentration in the purified fractions was determined using the Coomassie Plus Protein Assay kit (Pierce, Rockford, IL, USA). Bovine serum albumin was used as standard (bovine serum albumin, fraction V, Pierce, Rockford, IL, USA).

Molecular Weight Distribution

Molecular weight distributions of bioactive peptide fractions (crude and purified) were determined by size exclusion chromatography (SEC) on a Superdex Peptide HR 10/300 GL column (GE Healthcare, Baie d'Urfé, QC, Canada) using a FPLC system (AKTÄ Explorer 100, GE Healthcare, Baie d'Urfé, QC, Canada) based on the method described previously [4]. The mobile phase (isocratic) consisted of 50 mM sodium phosphate buffer containing 150 mM of NaCl at pH 7.0. Samples were eluted at a flow rate of 0.5 ml/min. The sample size loaded onto the SEC column was 50 µl, corresponding to approximately 50 µg

of proteins/peptides according to the total protein assay described previously. Protein hydrolysates analyzed on the Superdex Peptide HR 10/300 GL column were detected by monitoring absorbance at 215 nm.

Minimum Inhibitory Concentration Determination of the Purified Fraction

Inhibitory activity measurement of the purified fraction was performed using a critical microdilution method [10] with slight modifications. Briefly, the purified sample (200 μg ml^{-1}) was sterilized by filtration on a Millex PVDF Durapore 0.45-μm filter. Twofold serial dilutions of the sample were made in duplicate by repeated transfers of 125 μl in 125-μl volumes of Tryptic Soy Broth (TSB) supplemented with 0.08% cellobiose (w/v), 1.0% NaCl (w/v) and 2.0% Bacto-peptone (w/v) in a 96-well Falcon 3072 flat-bottom polystyrene microplate (Becton–Dickinson Labware, Franklin Lakes, NJ, USA). Each well was inoculated with 50 μl of an overnight culture of the indicator strain *V. vulnificus* LEA GN38 precisely adjusted at 2×10^5 CFU/ml. The microplate was placed in a Multiskan Ascent microplate reader (Labsystems, Helsinki, Finland) and incubated at 35°C for 16 h. Optical density (OD) was monitored at 600 nm every 10 min. The minimal inhibitory concentration (MIC) was defined as the lowest sample concentration that resulted in the absence of turbidity (OD_{600nm} equal to negative control) after 16 h of incubation.

The mode of action of the purified sample was assessed according to Tome et al. [40] by adding 35 μl of sample in the inoculated wells after 5 and 6 h during the exponential growth phase in the microplate reader, followed by regular OD_{600nm} readings.

Determination of Copper and Other Elements in Bioactive Peptide Fractions by Inductively Coupled Plasma-Mass Spectrometry

Metals and metalloids were measured in bioactive peptide fractions (crude and purified) diluted with acidified ultrapure water (NANOpure Infinity system, Barnstead Int., Dubuque, IO, USA) by inductively coupled plasma interfaced to a quadrupole mass spectrometer (ICP-MS, Agilent 7500c, Agilent, Japan) equipped with a micro flow nebuliser. The element quantification was performed in normal mode with a seven point's calibration plot of standard solution Multi-Element IV (Fluka Chemie Gmbh, Switzerland) with a concentration range from 0.125 to 200 ng ml^{-1} for the heavy metals, arsenic and selenium. Potassium, calcium and magnesium were quantified with a seven point's calibration plot of standard solution Trace metal III (JT Baker, Philisburg, NJ, USA) with a

concentration range from 31 to 1,000 ng ml^{-1}. Performance of the method was assessed by the analysis of a quality control (QC) solution of known metal concentration and a procedural blank. Control of the system, acquisition and data processing were carried out with the Agilent 7500 ICP-MS ChemStation$^®$ software (revision C).

Characterization of Organic Compounds in the Active Fraction by Electrospray Ionization-Mass Spectrometry (ESI–MS) and Liquid Chromatography-Electrospray Ionization-Mass Spectrometry

Structural information of the main compounds present in the active fraction was studied by mass spectrometry with electrospray ionization (ESI) with sample introduction by direct infusion or following chromatographic separation by liquid chromatography. The LC–MS system consisted of a liquid chromatograph Surveyor LC coupled via an atmospheric pressure ionization interface to an ion trap mass spectrometer with MS^n capabilities (Surveyor LC linked to a LCQ Advantage, ThermoFinnigan, San Jose, CA, USA). Direct infusion was achieved by diluting the active fraction in water and infusing directly the solution in the ESI source via the syringe pump. Detection of ions was performed in positive mode with a mass scan range from 60 to 2,000 amu. In MSMS (MS^2) experiment, the precursor ion ($m/z = 578$) was fragmented with a collision-induced dissociation energy of 40 (arbitrary unit) and the positive product ions were scanned from 155 to 600 amu. Chromatography was performed on a Discovery RP-C$_{18}$ 5 cm × 2.1 mm column (Supelco, Bellefonte, PA, USA), equipped with a pre-column, with an isocratic elution with 96% water and 4% acetonitrile in 6 min with a flow rate of 0.4 ml min^{-1}. Injection volume of the active fraction diluted in water was 10 μl in full loop mode. Detection of ions was performed in positive mode with a mass scan range from 100 to 2,000 amu. System control, data acquisition and data processing were performed with the Xcalibur 1.3 software (ThermoFinnigan, San Jose, CA, USA).

Qualitative Analysis of Copper Associated to Organic Compounds in the Active Fraction by LC-ICP-MS

Distribution of copper associated with organic molecules was determined using the same RP-C$_{18}$ column and chromatographic conditions as in liquid chromatography-electrospray ionization-mass spectrometry (LC–ESI–MS), on a LC1100 liquid chromatograph (Agilent, Japan) coupled to the Agilent 7500c ICP-MS equipped with a micro flow nebuliser. The signal at a mass-to-charge ratio of 63 was monitored for the detection of copper. Control of the system, acquisition and data processing were carried out

with the Agilent 7500 ICP-MS ChemStation® software
(revision C).

Results

Detection of Antibacterial Activity in Enzymatic Hydrolyzed Snow Crab By-Product Fractions

All snow crab hydrolysate fractions previously produced
[4] were analyzed for the determination of their inhibitory
spectrum against bacterial strains of interest in environ-
mental, food and health sectors. On the basis of clear zones
obtained by the agar well diffusion assay, active peptide
fractions were selected (data not shown). Mainly, low
molecular weight fractions corresponding to nano-filtration
retentate (ranging from 200 Da to 1 kDa) exhibited anti-
bacterial activity against several bacteria. Growth inhibi-
tion of many Gram-negative bacteria, such as *Aeromonas
caviae*, *Aeromonas hydrophila*, *Campylobacter jejuni*,
Listonella anguillarum, *Morganella morganii*, *Shewanella
putrefasciens*, *Vibrio parahaemolyticus* and *Vibrio vulnifi-
cus*, was noted as well as for a few Gram-positive bacteria,
such as *Listeria monocytogenes*, *Staphylococcus epider-
midis* and *Streptococcus agalactiae* (Table 1).

Bioactive Peptide Fraction Analyses

The antibacterial peptide fraction (nano-filtration retentate)
was characterized to determine its chemical composition
(moisture, ash, crude proteins and lipids). As seen in a
previous work [4], this fraction is comprised mainly of
proteins and minerals (74.3 and 15.5%, respectively),
expressed on a dry weight basis, and lipids were not
detected.

The antibacterial peptide fraction was also analyzed for its
amino acid content. The total amino acids measured in the
nano-filtration retentate fraction were determined in previ-
ous work [4]. The three most important amino acids were
arginine (4.6%), glutamic acid (5.3%) and tyrosine (4.8%)
residues, which are amino acids with polar side chains
(including not charged and charged). The content of residues
with polar side chains was higher (27.0%) than that of other
amino acid residues with non-polar side chains (23.5%).

An absence of inhibition zone in the agar diffusion assay
was noticed at pH 5. All other pH values demonstrated the
presence of inhibition zone. However, antibacterial activ-
ities increased at alkaline pH (Table 2).

Purification Process

The initial developmental work for the purification process
was performed using 1-ml resin columns. Several ion

Table 2 Results for agar well diffusion assay using the indicator
strain *V. vulnificus* LEA GN38

Peptide fraction	Activity detected (mm)
Fraction adjusted at pH 5	0
Fraction adjusted at pH 6	8
Fraction adjusted at pH 7	9
Starting fraction at pH 8	10
Fraction adjusted at pH 9	12

^a Activity was detected by the diameter measurement (mm) of the
inhibition zones in the agar diffusion

exchange chromatography (IEX) and hydrophobic inter-
action chromatography (HIC) resins were tested as
described previously. Screening of various resins permitted
to develop the peptide purification process which could
then be used at a larger scale using 20-ml resin XK16/20
columns in order to generate more bioactive peptide
material. IEX and HIC using SP-Sepharose^{TM} Fast Flow
cation exchange column and Phenyl Sepharose^{TM} High
Performance hydrophobic interaction column appeared as
promising purification and polishing steps. Because of the
ionic resin used in this study (SP-Sepharose); these anti-
bacterial peptides, recovered in the flow through, seem to
possess anionic properties, which are considerably less
frequent than cationic properties for these kinds of peptides
[28].

Purification results are given in Table 3. The total pro-
tein concentration in the starting material (580 ml) was 323
μg ml^{-1}. According to Table 3, approximately 12% of total
proteins were lost during the first purification step. Only
7% of total proteins could be recovered after the last
purification step. However, even if the total protein con-
centration was low (50 μg ml^{-1}), biological activity shows
an improved inhibition zone (diameter of 10 mm) in
comparison with the SP-Sepharose step (diameter of
9 mm), which could indicate that the active peptides may
have been concentrated. Only the purified antibacterial
peptide fraction was analyzed for MIC determination, and
the value obtained was 25 μg ml^{-1}.

Figure 1 shows the effect of bioactive peptide fraction
(Phenyl-Sepharose eluate (248 μg ml^{-1})) on the growth of
V. vulnificus LEA GN38. The addition of 35 μl of the
peptide fraction in the wells after 5 h of incubation stopped
the growth but did not decrease the optical density at
600 nm (OD$_{600nm}$). However, the peptide fraction addition
in the wells after 6 h rapidly caused a drop in the OD$_{600nm}$
for approximately 1 h and then caused complete growth
inhibition.

Figure 2 shows the FPLC profile obtained for all puri-
fication steps using size exclusion chromatography. The
FPLC profile of peptide fraction loaded on the IEX chro-
matography column: (a) was characterized by the presence

Table 3 Purification scheme for the antibacterial peptide obtained from snow crab by-products

Purification step[a]	Volume (ml)	Protein (μg/ml)[b]	Total protein (mg)	Activity detected[c] (mm)	Activity detected[d] (μg/ml)	Recovery per step (%)
Antimicrobial peptide fraction	580	323	187	11	NA	100
SP-Sepharose[TM] fast Flow eluate (flow through)	1,046	158	165	9	NA	88
Phenyl-Sepharose[TM] High performance eluate	275	50	14	10	25	7

[a] Several small-scale purification trials were first done using 1-ml columns and then scaled-up to columns of larger volumes (20 ml). Results presented here correspond to the larger scale purification (repeated several times)

[b] Total protein

[c] Activity was detected by the diameter measurement (mm) of the inhibition zones in the agar diffusion

[d] Activity was determined using the minimum inhibitory concentration (MIC)
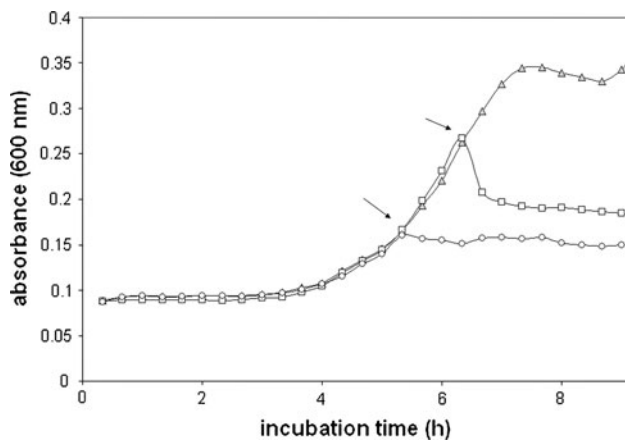
NA not analyzed

Fig. 1 Effect of adding 35 μl of the partially purified peptide (Phenyl-Sepharose eluate (248 μg/ml)) on the growth of *V. vulnificus* LEA GN38, after 5 (*circles*) and 6 (*squares*) h of incubation; *triangles* represent control growth of the tested microorganism. Data points represent the average of three experiments run in duplicate

of low molecular weight molecules around 800 Da and less. The presence of low molecular weight proteins could be explained by the enzymatic hydrolysis treatment performed during the processing of snow crab by-products [4]. After a first purification step using an IEX column, the FPLC profile was quite similar to the initial one (b) but was characterized by lower protein amounts (Table 3), which could explain the weaker inhibition zone in (Table 3). Finally, the results of the last purification step, using HIC, showed only one peak around 800 Da on the FPLC profile (c), which was active as indicated in Table 3.

Mineral Determination

Following the purification method development, minerals were also analyzed. Minerals constitute an important portion of the starting antibacterial peptide fraction (15.5%, expressed on a dry weight basis) [4]. Table 4 shows minerals present in the antimicrobial peptide fractions following the purification process. The initial antibacterial
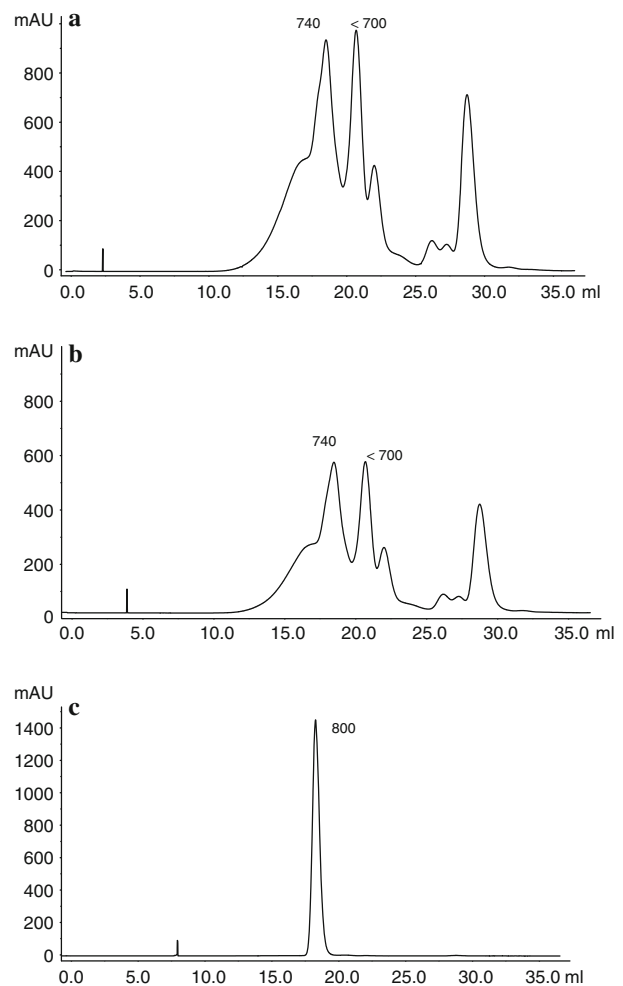

Fig. 2 SEC-FPLC chromatograms of antibacterial peptide fractions during the purification process by monitoring absorbance at 215 nm. **a** antimicrobial peptide fraction, **b** SP-Sepharose eluate, **c** Phenyl-Sepharose eluate. The value marked for each peak was expressed in Da (mAU = arbitrary units of absorbance)

peptide fraction was rich in K (154 μg g^{-1}), Mg (147 μg g^{-1}) and Cu (65 μg g^{-1}). All mineral contents decreased following each purification steps, but the Cu

Table 4 Minerals in antibacterial peptide fractions following the purification process. Concentration is expressed as μg g^{-1} of liquid fraction

Minerals	Antimicrobial peptide fraction	SP-Sepharose^{TM} fast flow eluate (flow through)	Phenyl-Sepharose^{TM} high performance eluate
V	0.26 ± 0.01	0.18 ± 0.01	0.08 ± 0.01
Mn	0.26 ± 0.01	0.16 ± 0.01	0.010 ± 0.001
Fe	12.84 ± 0.64	7.31 ± 0.26	ND
Ni	6.75 ± 0.07	4.45 ± 0.11	0.010 ± 0.001
Co	1.80 ± 0.02	1.21 ± 0.01	0.010 ± 0.001
Cu	65.72 ± 0.66	27.44 ± 0.32	3.25 ± 0.03
Zn	16.68 ± 0.17	7.71 ± 0.04	0.11 ± 0.01
As	36.71 ± 0.37	26.41 ± 0.18	0.07 ± 0.01
Se	5.16 ± 0.08	3.60 ± 0.04	0.020 ± 0.002
Cd	0.51 ± 0.01	0.30 ± 0.01	ND
Pb	0.13 ± 0.01	0.03 ± 0.01	0.010 ± 0.001
Mg	147.0 ± 0.7	11.0 ± 0.1	0.10 ± 0.01
K	154.0 ± 4.6	113.0 ± 0.1	0.3 ± 0.1
Ca	36.0 ± 0.2	19.0 ± 0.6	0.2 ± 0.1

Results are mean values of three replicates ±SD for V to Pb and five replicates ±SD for Mg, K and Ca

ND not detected

content recovered within the purified fraction was higher at 3.25 μg g^{-1} in comparison with other minerals, suggesting a possible role of this metal in antibacterial properties. However, it is unlikely that the antibacterial activity observed was caused solely by the presence of copper in solution, because no antibacterial activity was detected when a copper sulfate solution ($CuSO_4$), at the same concentration, was tested (data not shown).

Characterization of Active Fraction by Mass Spectrometry with Electrospray Ionization

The total ion current chromatogram obtained from the qualitative analysis of the purified active fraction by LC–ESI–MS shows a main peak eluting at 0.77 min (Fig. 3a, upper chromatogram), suggesting a polar compound with low affinity for the reversed phase. However, two compounds are revealed with ion chromatogram traced with two major ions detected with mass-to-charge ratios of 578 and 173 (Fig. 3a, middle and lower chromatograms, respectively). Thus, the active fraction would be comprised of a polar compound eluting at 0.8 min and a more hydrophobic molecule, with a greater affinity to the non-polar C_{18} stationary phase than to the aqueous solvent at 96%, eluting at 3 min with a large tailing due to the isocratic conditions. As no unambiguous structural information from the mass spectra at both retention times was evidenced, we cannot exclude that these two chromatographic peaks correspond to two groups of organic molecules, with different polarity, that form clusters in the ion source. The direct infusion in the ESI source of the active peptide fraction produced a mass spectrum of positive ions dominated by a repetitive fragmentation pattern (Fig. 4a). The heavier ion in the mass spectrum has a m/z ratio of 1873, and thereafter, a successive loss

of 185 is observed down to the ion with a mass of 208. By isolating the ion with $m/z = 578$ as the precursor ion in MS^2 mode, we observed the loss of two 185 units $578 \rightarrow 393$ and $393 \rightarrow 208$ (Fig. 4b). A good candidate to the identity of the 185 unit is the sodium adduct of a hexose $(162 + 23)$ [10]; as discussed by these authors, an oligosaccharide coordinated with a sodium ion will fragment along three main pathways: loss of the metal ion, glycosidic bond cleavage and cross-ring cleavage. The transition $578 \rightarrow 415$ illustrates the loss of a hexose, whereas the transitions $415 \rightarrow 393$ and $230 \rightarrow 208$ illustrate the loss of a Na^+ cation. The cross-ring cleavage produces a loss of an ion with $m/z = 127$ (e.g. $578 \rightarrow 451$, Fig 4a) and also the loss of an ion with $m/z = 58$ (e.g. $451 \rightarrow 393$, Fig 4a). The ion with $m/z = 208$ could correspond to a hexose with two sodium atoms as the ESI process is known to favor sodium adduct formation over protonation if background alkali concentration is important [25].

Copper is the main metal found in the active peptide fraction (Table 4). The qualitative analysis by liquid chromatography coupled to ICP-MS shows that copper is associated with the more polar compound or group of compounds, detected under the same chromatographic conditions by LC–ESI–MS (Fig. 3b) at retention time of 0.8 min. The ionization process in the argon plasma of the ICP-MS destroys completely the molecular structure leaving only ionized elements, thus the signal monitored with $m/z = 63$ can only be attributed to Cu^+. No copper was detected within the time interval corresponding to the more hydrophobic group of molecules. Hence, the bioactive peptide fraction purified according to our purification strategy contains amino acids, as explained earlier, but also copper and two groups of organic molecules with different polar characteristics.
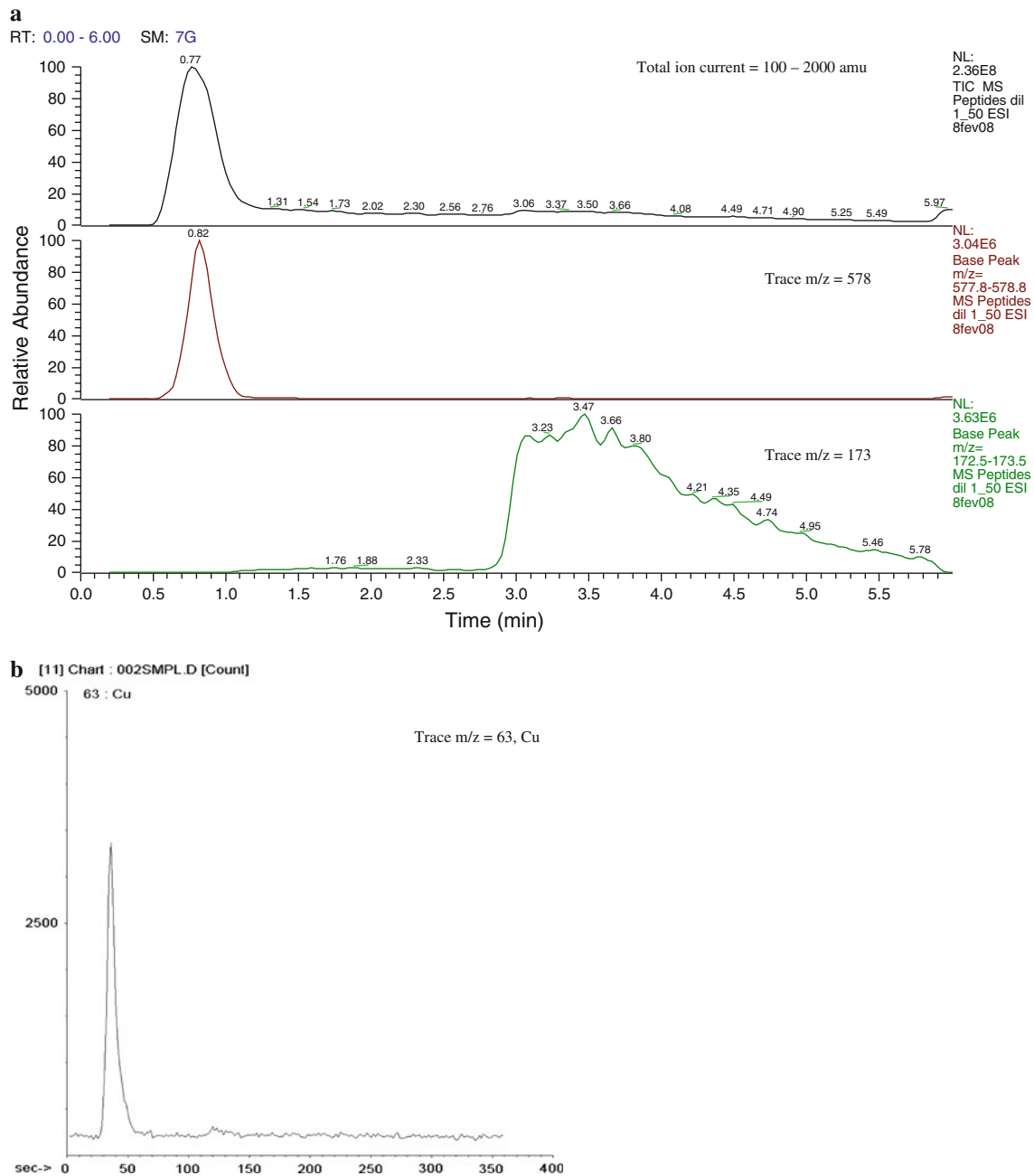
Fig. 3 LC-MS chromatograms, on a RP-C18 column, of the active fraction diluted 1:50 in water. **a** LC–ESI–MS, **b** LC-ICP-MS

Discussion

Detection of Antibacterial Activity in Enzymatic Hydrolyzed Snow Crab By-Product Fractions

Investigations into potential utilization of the fractions produced by enzymatic hydrolysis of snow crab by-products were undertaken in the present study in order to identify and characterize AMPs. A main fraction composed of low molecular weight molecules exhibited antibacterial activity against several bacteria. A major activity was detected against Gram-negative bacteria (*A. caviae*, *A. hydrophila*, *C. jejuni*, *L. anguillarum*, *M. morganii*, *S. putrefasciens*, *V. parahaemolyticus* and *V. vulnificus*), while the growth of only a few Gram-positive bacteria was inhibited (*L. monocytogenes*, *S. epidermidis* and *S. agalactiae*). The bacterial strain *V. vulnificus* was chosen as indicator strain due to the higher and clearer inhibition detected and reproducibility. In addition, *Vibrio* such as *V. vulnificus* and *V. parahaemolyticus* are pathogenic bacteria causing food poisonings. Moreover, the bacterial species *Vibrio* being widespread in the marine environment
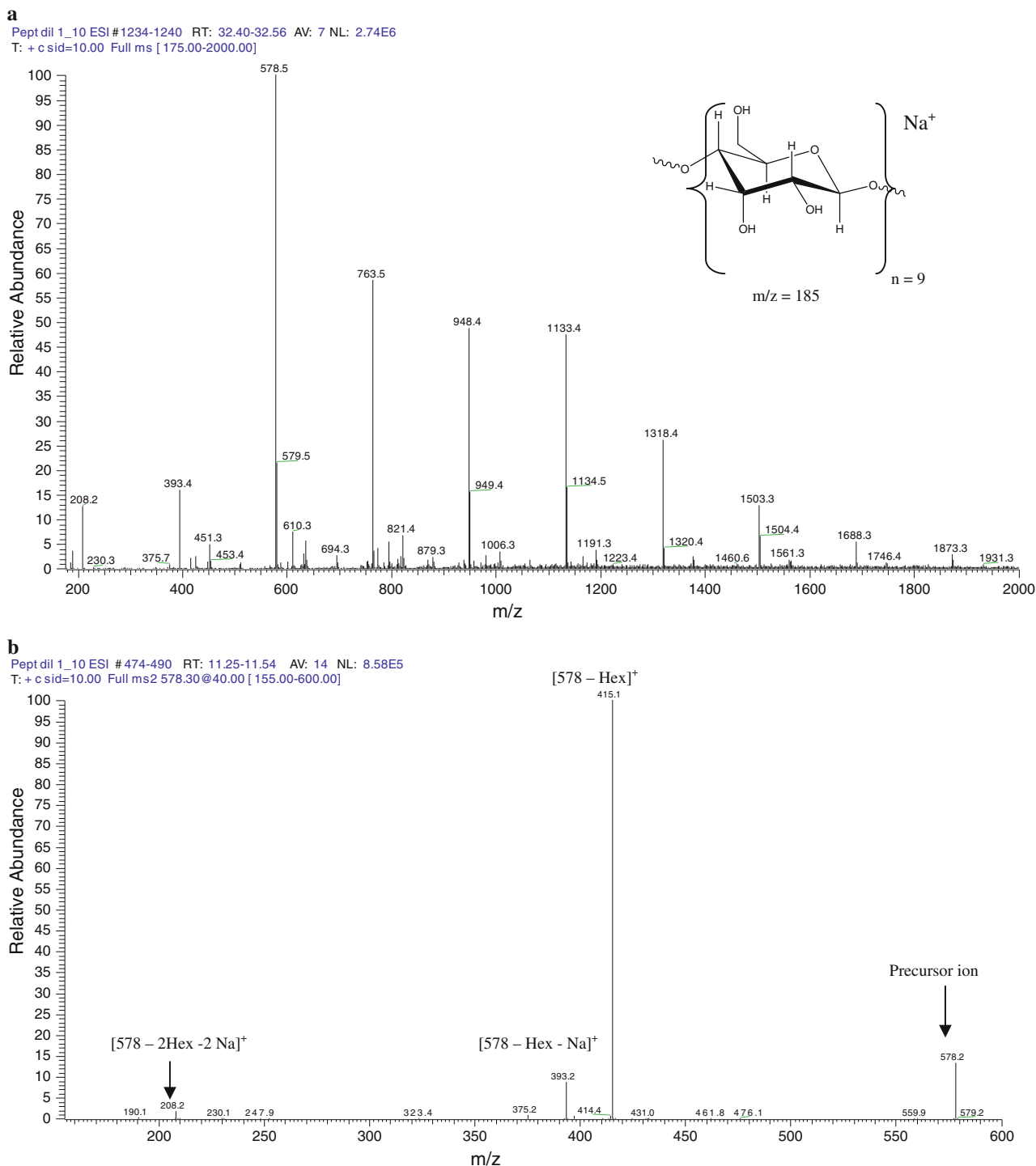
a

Pept dil 1_10 ESI #1234-1240 RT: 32.40-32.56 AV: 7 NL: 2.74E6
T: + c sid=10.00 Full ms [175.00-2000.00]

b

Pept dil 1_10 ESI # 474-490 RT: 11.25-11.54 AV: 14 NL: 8.58E5
T: + c sid=10.00 Full ms2 578.30@40.00 [155.00-600.00]

Fig. 4 Mass spectrum of the active fraction obtained by electrospray ionization (ESI) with direct infusion of the diluted sample. **a** fullscan mode with mass range 175–2,000 amu; **b** MS^2 experiment with the fragment 578 as the precursor ion

and several shell diseases in crustaceans are attributed to *Vibrio* species [29]. It is possible that the snow crab could develop a defense mechanism against these bacteria. Such biological activity has already been postulated for peptides resulting from the spider crab (*Hyas araneus*) from the cool

waters of Norway [18] as well as for peptides from blue crab (*Callinectes sapidus*) from the Atlantic Ocean [23]. Furthermore, the microbial flora identified in certain crabs from cool water is composed of *Vibrio* bacterial strains [14].

Bioactive Peptide Fraction Analyses

The bioactive peptide fraction (nano-filtration retentate) was characterized and was mainly composed of proteins and minerals (74.3 and 15.5%, respectively). According to amino acids analysis, this fraction would contain a strong proportion of amino acids with polar side chains charged (glutamic acid and tyrosine) and uncharged (arginine). AMPs vary substantially in their amino acid sequences. In spite of variations in structure and size, the majority of antimicrobial peptides are amphiphilic, displaying both hydrophilic and hydrophobic surfaces [39].

Cationic antimicrobial peptides are known to possess small numbers of acidic residues (glutamate or aspartate) and excess numbers of cationic (arginine or lysine and/or histidine) residues and around 30–50% hydrophobic residues [16]. Anionic antimicrobial peptides, which are less known in comparison with cationic peptides, possess a considerable amount of acidic residues. The presence of aspartic acid would play an important role in biological activity [9]. Among the major classes of AMPs, there are peptides with an overrepresentation of one or two amino acids [39], which include peptides rich in proline, glycine, cysteine, histidine or tryptophan [28]. Also, the amino acid composition dictates the charge of a protein or a peptide at a given pH. As seen for the bioactive peptide fraction, the biological activity increased at alkaline pH (from 6 to 9 values).

Purification Process and Purified Fractions Analysis

After identifying sensitive bacterial strains and following an initial biochemical characterization of the antibacterial peptide fraction, development of specific purification methods was pursued in order to isolate bioactive peptides. Antibacterial peptides present in this fraction were initially from a complex medium, and a purification step was required to remove other peptides and to minimize the loss of peptides of interest as well as preserving their biological activity. In the case of unknown peptides such as snow crab AMPs, the purification work requires finding the appropriate resin, pH and polarity conditions. AMPs characteristics are known to be mainly cationic and less often anionic, as well as hydrophobic or amphipathic. Bioactive peptide fraction analyses, presented earlier, have helped to establish preliminary conditions.

The resin selected for the purification of snow crab AMPs was the SP-Sepharose[TM] Fast Flow, a cation exchange resin, which allowed for the separation of cationic and anionic peptides. Active peptides, recovered in the flow through, were then injected on a Phenyl Sepharose[TM] High Performance hydrophobic interaction column. Following this purification scheme, bioactive peptides

seemed to possess anionic and hydrophobic properties. The last purified fraction showed an antimicrobial effect against *V. vulnificus* LEA GN38 strain, corresponding to a MIC value of 25 µg ml^{-1}, and this effect was due specifically to the presence of molecules of low molecular weight (around 800 Da). The MIC value obtained in the present study is lower in comparison with MIC values obtained for other AMPs originating from hydrolysates of oyster [26], which were most sensitive for Gram-positive bacteria, with MIC values between 40 and 60 µg ml^{-1}.

Other studies have been pursued in order to elucidate the structure and mechanism of action of snow crab AMPs. For instance, the LC–ESI–MS analysis of the active purified fraction demonstrated the presence of more than one peak. It appears that the active peptide fraction is a mixture of amino acids and other organic compounds. Mineral content results in the active fraction demonstrated a potential role of copper in antibacterial properties. The nature of minerals could be important for the antibacterial activity. For instance, the need for co-factors, such as Zn, Cu, Fe and Co, for biological activity is often required for anionic peptides [8, 9]. Since the active peptide fraction was extracted from crustacean biomass and copper is the main metal in the purified fraction (Table 4), one hypothesis could be that the active peptide fraction consisted of glycosylated hemocyanin fragments [21]. Hemocyanins are known as respiratory proteins in the form of metalloproteins containing two copper atoms that reversibly bind a single oxygen molecule (O_2) [5]. In addition, some peptide fragments generated from the C-terminal part of crustacean hemocyanin have been shown to possess antimicrobial activities [12, 24]. In most of the crustacean species studied, the antimicrobial activity has been located in the hemolymph and/or in the haemocytes, which were included in the snow crab by-products hydrolysates [4].

Further studies are necessary in order to understand the structure and mechanism of action involved in the antibacterial activity of the active peptide fraction, the identity of the organic molecules involved and the role of copper. The results obtained in this work show, for the first time, that snow crab by-products clearly contain molecules possessing a significant antibacterial activity. The field of study represented here holds the promise for developing new AMPs originated from snow crab by-products. Finding new anti-infectious agent offers a different biocidal mechanism to conventional antibiotics, which are becoming ineffective against the spread of multidrug-resistant pathogens. They can have many applications in public health such as biopreservatives in food, in animal feed, as well as in the medical area.

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