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Combining multidimensional liquid chromatography and MALDI–TOF-MS for the fingerprint analysis of secreted peptides from the unexplored sea anemone species *Phymanthus crucifer*

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A B S T R A C T

Sea anemones are sources of biologically active proteins and peptides. However, up to date few peptidomic studies of these organisms are known; therefore most species and their peptide diversity remain unexplored. Contrasting to previous venom peptidomic works on sea anemones and other venomous animals, in the present study we combined pH gradient ion-exchange chromatography with gel filtration and reversed-phase chromatography, allowing the separation of the 1–10 kDa polypeptides from the secretion of the unexplored sea anemone *Phymanthus crucifer* (Cnidaria/Phymanthidae). This multidimensional chromatographic approach followed by MALDI–TOF-MS detection generated a peptide fingerprint comprising 504 different molecular mass values from acidic and basic peptides, being the largest number estimated for a sea anemone exudate. The peptide population within the 2.0–3.5 kDa mass range showed the highest frequency whereas the main biomarkers comprised acidic and basic peptides with molecular masses within 2.5–6.9 kDa, in contrast to the homogeneous group of 4–5 kDa biomarkers found in sea anemones such as *B. granulifera* and *B. cangicum* (Cnidaria/Actiniidae). Our study shows that sea anemone peptide fingerprinting can be greatly improved by including pH gradient ion-exchange chromatography into the multidimensional separation approach, complemented by MALDI–TOF-MS detection. This strategy allowed us to find the most abundant and unprecedented diversity of secreted components from a sea anemone exudate, indicating that the search for novel biologically active peptides from these organisms has much greater potential than previously predicted.

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1. Introduction

1.1. The number of genomic/transcriptomic and proteomic/peptidomic studies of sea anemone toxins remains limited

Peptide toxin discovery has been traditionally approached by bioassay-guided chromatographic fractionation and further characterization of bioactive molecules [1]. So far the number of peptides sequences reported is extremely inferior to the 20 million peptides [2] estimated for all animal venoms combined; therefore most of their chemical diversity remains uncharacterized [3]. Aiming to shorten this difference, many recent genomic, transcriptomic, proteomic, and peptidomic studies of snakes, spiders, scorpions and cone snails, have sped up the discovery of new peptides and proteins.

However, the application of these approaches to other venomous animals such as sea anemones still remains limited, even though these organisms are well known sources of peptide toxins [4–11] (ion channel toxins, protease inhibitors, peptide cytolysins) and protein toxins [12,13] (protein cytolysins and phospholipases A2), of pharmacological and therapeutic interest. The applications of genomic/transcriptomic approaches [14–17] to the study of sea anemone toxins have been accelerated since 2006, from major studies of *Nematostella vectensis* [18,19] and *Anemonia viridis* [20], and very recently from *Bunodosoma granulifera* [17] using for the first time the high-throughput 454 pyrosequencing technology in the study of sea anemones. Besides the genomic/transcriptomic analyses, a very limited number of peptidomic studies focused on

Abbreviations: IEC, ion-exchange chromatography; RP-HPLC, reversed-phase high performance liquid chromatography; MALDI–TOF-MS, matrix-assisted laser desorption/ionization-time of flight mass spectrometry; RPC18, reversed-phase octadecyl stationary phase.

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sea anemones toxins has been performed, in *Bunodosoma cangicum* [21], *B. granulifera* and *Stichodactyla helianthus* [17].

Specialized sea anemone (protein or DNA) sequences databases barely exist, only StellaBase, the *N. vectensis* Genomics Database [18]. Genomic data can also be found in general DNA DataBanks (GenBank, DDBJ and EMBL) whereas transcriptomic data (from *A. viridis*) is located in the GenBank division dbEST-NCBI, the Expressed Sequence Tags database. Sea anemone protein/peptide sequences are annotated under the Animal Toxin Annotation Program (formerly Tox-prot) [22] of the general database UniProtKB/Swiss-Prot, also in the animal toxin metadatabase ATDB [23]. By contrast, other animal toxins have been grouped into specialized databases such as ArachnoServer [24] and ConoServer [25]. More recently, a review on animal toxins databases was published [26].

1.2. Some limitations of previous fingerprinting analyses of sea anemone secretions

As mentioned above, few peptidomic studies of sea anemones venoms have been reported [17,21], comprising profiling and structural characterization of novel peptides by Edman sequencing and transcriptomic analyses. The separation and detection strategy employed in these studies allowed finding a large number of peptides; however several factors may have led to an underestimation of this overall peptide complexity contained in sea anemone secretions.

a) Short molecular mass range covered by gel filtration
   Previous fingerprinting analyses of sea anemone secretions [17,21] did not exploit the whole peptidomic range, comprising 1–10 kDa fractions from gel filtration chromatography. Instead, a shorter range was collected, 2–5 kDa, defined as the neurotoxic fraction [27]. However several classes of biologically peptides from sea anemones fall outside that mass range, such as protease inhibitors, type 2 potassium channel toxins, cytolytic peptides and small crab-paralyzing toxin. These classes of toxins are likely to be less represented when only those fractions within 2–5 kDa are considered for subsequent analyses.
b) Chromatographic separation of peptides only relaying on RP-HPLC
   Several peptidomic studies of other venomous animals [28–31], such as scorpions and spiders, have used multidimensional chromatographic approaches combining ion-exchange and reversed-phase chromatographic steps. The introduction of ion-exchange chromatography (IEC) greatly improves peptide separation and detection, given the high loading capacity of ion exchangers and resolution power of the technique according to electrical charge differences among peptides, representing a complement to reversed-phase separations, which are based on a different mechanism [32]. The chromatographic separation strategy employed in previous peptidomic analyses of sea anemones [17,21] comprised a combination of gel filtration and reversed-phase chromatography for the study of the neurotoxic fractions from the sea anemones *Bunodosoma cangicum*, *S. helianthus* and *B. granulifera*. Neither salt gradient IEC nor pH gradient IEC was used, contrasting to peptidomic studies of other animal venoms [28–31] and the bioassay-guided isolation of sea anemone toxins employing salt gradient IEC [34–43]. Moreover, to our knowledge, pH gradient IEC has not been employed in any peptidomic analysis of animal venoms.
c) Reduced number of fractions included in MS analyses
   Previous studies of sea anemone venoms [17,21] considered a limited number of reversed-phase fractions for MS analyses, comprising only those manually collected according to UV detection. Consequently many low-abundance peptides may have escaped from fingerprinting analysis, especially if they were present in very small peaks, valley between peaks and flat parts of the chromatographic profile.

Given the existence of few peptidomic reports on sea anemones species, their limitations for peptide separation and detection, as well as the large number of unexplored species, the present work employed a multidimensional chromatographic approach combined with MALDI–TOF detection for peptide fingerprinting analysis of the secretion extracted from the unexplored species *Phymanthus crucifer* (Cnidaria/Phymanthidae). Our separation strategy comprised the isolation of secreted 1–10 kDa peptides using gel filtration chromatography, and subsequent fractionation by pH gradient IEC and reversed-phase high performance liquid chromatography (RP-HPLC). The chromatographic data and molecular masses measured by MALDI–TOF-MS, allowed the construction of venom maps composed of hundreds acidic, neutral and basic secreted peptides. This is the first report on the peptide fingerprint of a sea anemone belonging to the family Phymanthidae. Our strategy improved peptide separation and detection, showing that sea anemone secretion is more complex than previously estimated [17,21].

2. Materials and methods

2.1. Peptide extraction and chromatography

Nineteen specimens (204 g) of the sea anemone *P. crucifer* were collected at the northeast coast of Havana, Cuba, and carried to the laboratory. Immediately the mucus was extracted, mainly based on a previous study [44]. All sea anemone specimens were placed on a funnel and 200 mL distilled water were added in 40–50 mL portions during 5 min; the secreted mucus (sample #1) was simultaneously collected. For a more exhaustive extraction all the specimens were subsequently placed into a 1 L beaker containing 200 mL distilled water, during 5 min. The secreted mucus (sample #2) was separated from the specimens and mixed with sample #1.

Subsequent gel filtration and cation-exchange chromatographic steps were performed with Biorad low pressure chromatographic equipment (Biorad, USA), composed of a pump, UV/conductivity detector, fraction collector and paper recorder.

2.1.1. Gel filtration

A Sephadex G-50 gel filtration column (Pharmacia, Sweden), 5 cm × 93 cm, was calibrated with molecular mass markers: ovalbumin (43 kDa), trypsin inhibitor (20 kDa), cytochrome C (12.4 kDa) and bradykinin (1.24 kDa). Log M_r vs. V_R plot [45], linear regression and calculations to estimate the retention volumes (V_R) corresponding to the molecular mass range 1–10 kDa, were done in Microsoft Excel 2007 (Microsoft, USA). The lyophilized sea anemone sample was dissolved in 0.1 M ammonium acetate, centrifuged at 4000 × *g* and loaded onto the Sephadex G-50 M. The separation was performed at the flow rate of 2 mL/min; 125 fractions of 20 mL each were online monitored at 280 nm and collected.

2.1.2. pH gradient ion-exchange chromatography (pH gradient IEC)

A 500 mL stock solution composed of the following buffering compounds (AppliChem, Germany) at 0.015 M each was prepared: Tris (hydroxymethyl)aminomethane (pK = 8.20), acetic acid (pK = 4.76), *o*-phosphoric acid (pK_1 = 2.12, pK_2 = 7.20, pK_3 = 12.32), histidine (pK_1 = 1.82, pK_2 = 6.00, pK_3 = 9.17) and aspartic acid (pK_1 = 2.09, pK_2 = 3.86, pK_3 = 9.82). The stock solution was used to prepare the working buffers (0.005 M each compound) at defined pH values, which for practical reasons were called BMpH, indicating the buffer mixture adjusted at a certain pH value.

A volume of 100 mL from the stock solution was diluted with water up to approximately 250 mL, and adjusted at pH 3 with 1 M HCl, using a pH/Cond 340i set multimeter (Omnilab, Germany) for pH measurement. After pH adjustment the buffer mixture was filled with water up to the final volume of 300 mL. This buffer mixture at pH 3 was called BM3. Similarly, 300 mL of the buffer mixture at pH 12 (BM12) was prepared from the stock solution, and the pH adjusted with 1 M NaOH.

The fractions eluting within the mass range 1–10 kDa from Sephadex G-50 were pooled, concentrated by vacuum in a rotary evaporator and desalted in Sephadex G-10 (4 cm × 48 cm) at 2 mL/min, equilibrated with 0.005 M ammonium acetate (p.a, Merck, Germany). The sample (1–10 kDa polypeptides) was acidified to pH 4 and applied to the Fractogel EMD SO_3^- 650 M (Merck, Germany) cation-exchange column, 1.8 cm × 5 cm. Non-retained compounds were washed out from the column using 0.005 M ammonium acetate at pH 4. Then the column was equilibrated with 50 mL BM3. The retained peptides were eluted at a flow rate of 1 mL/min using a pH gradient from BM3 (250 mL) to BM12 (250 mL), generated in a gradient mixer GM-1 (Pharmacia, Sweden). This 500 mL gradient (39 column volumes) in 500 min, comprising 250 mL BM3 + 250 mL BM12, is equivalent to an ascending gradient of 0.2%/min, from 100% BM3 to 100% BM12 at 1 mL/min in current automated equipments.

One hundred fractions of 5 mL each were online monitored at 280 nm, collected and manually read using the pH/Cond meter. Twelve pools of chromatographic fractions (P1 to P12) were made up according to the chromatographic profile; those having pH values above 7 were acidified with acetic acid.

2.1.3. *Reversed-phase high performance liquid chromatography (RP-HPLC)*

P1 to P12 were submitted separately to reversed-phase chromatography in a Bondapak RP-C18 column (Waters, USA) of dimensions 10 mm × 250 mm, previously equilibrated with solvent A, 0.1% trifluoroacetic acid (TFA) in water. Elution was carried out at a flow rate of 2.5 mL/min, using the gradient sequence 2–52% B in 50 min, 52–95% B in 5 min and a washing step with 95% B, being solvent B 0.05% TFA in acetonitrile. Eluting compounds were detected at 280 nm. Fractions were automatically collected every one minute. RP-HPLC separations were performed in a BioCAD SPRINT system (PerSeptive Biosystems, USA). The retention of a peptide expressed as percentage of acetonitrile (%ACN) was estimated similarly to our previous report [17], according to $\%ACN_e = \%ACN_0 + (\Delta\%ACN/t_G) \times (t_R - t_0 - t_D)$ [46], being $t_0 = 5$ min, $t_D = 0.5$ min, $\Delta\%ACN/t_G = 1\%/\text{min}$), $ACN_0 = 0\%$. Considering the initial isocratic step at 0% ACN during 2 min, a $t_{delay} = 2$ min was introduced in the calculation so $\%ACNe = 0\% + 1\%/\text{min} \times (t_R - 7.50\ \text{min})$.

The proteinaceous contents from the sea anemone secretion, gel filtration and cation-exchange chromatographic fractions were estimated with the bicinchoninic acid colorimetric assay [47], using a BCA Kit (AppliChem, Germany) and Bovine Serum Albumin (BSA) as protein standard.

2.2. *Molecular mass measurements and peptide maps*

Molecular mass analysis of RP-HPLC fractions was performed with a Voyager DE Pro matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI–TOF–MS) instrument (1.2-m Xight tube, 337-nm laser; Applied Biosystems, Darmstadt, Germany). Measurements were performed in linear mode; the mass resolution of the instrument was 4000. The matrix solution was prepared with α-cyano-4-hydroxycinnamic acid dissolved in mass buffer (50% acetonitrile + 0.1% TFA) up to saturation. One microliter of sample solution and matrix solution were mixed on a 100-well stainless steel multiple-sample tray. The crystallization process was accelerated by air-drying using a microventilator. Ions were accelerated at 25 kV, and up to 50 laser shots were automatically accumulated per sample position. Biospectrometry Workstation 5.1 was used as controlling software.

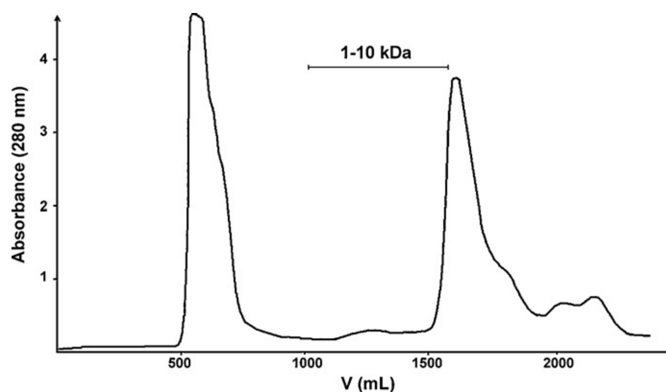

Fig. 1. Gel filtration profile of the *P. crucifer* secretion in Sephadex G-50M (5 cm × 93 cm). 0.1 M ammonium acetate was used as eluent at a flow rate of 2 mL/min. Detection was monitored at 280 nm. Fractions of 20 mL each were collected; those within the 1–10 kDa mass range were pooled.

External mass calibration of the instrument was carried out with the peptide standards human bradykinin (Mr 1060.2), human secretin (Mr 3039.4) and human PTH 1-38 (Mr 4456.0). Sample measurements were performed in triplicate, with an average mass accuracy lower than 100 ppm.

Secretion peptide maps and the histogram were constructed by using Microsoft Excel 2007 (Microsoft, USA) and the statistical software Origin 6.0 (Microcal Software, MA, USA), respectively.

3. Results

3.1. *Chromatographic separation of the sea anemone peptides*

The immersion of *P. crucifer* specimens in distilled water yielded a total protein content of 742 mg (average: 39 mg/specimen). The resulting sample was divided into two identical portions (371 mg each) that were individually applied onto the Sephadex G-50 column; the chromatographic profile is shown in Fig. 1. The polypeptides of our interest (approximate range 1–10 kDa) were located between fractions 52 and 83 ($V_R = 1020$–1660 mL) according to the calibration curve ($\log M_r = -0.00143 \times V_R + 5.43459$; $R^2 = 0.993$) using molecular mass standards. Compounds with molecular masses outside the selected range, such as proteins, small organic compounds and salts were discarded.

Gel filtration fractions (52–83) within the 1–10 kDa mass range were pooled and lyophilized. The peptide pool (89 mg, 12% of the total proteinaceous content) was desalted and submitted to ion-exchange chromatography in Fractogel EMD SO_3^- 650 M, using a pH gradient over the wide pH range 3–12 (from BM3 to BM12) for the retention and fractionation of most groups of acidic and basic peptides present in the *P. crucifer* secretion (Fig. 2A and B). All collected fractions eluted within the linear pH range of the separation, 3.5–10.8, $R^2 = 0.993$ (Fig. 2A), and were grouped into twelve pools, P1–P12 (Fig. 2B), within the following pH ranges: P1 (4.00–5.08), P2 (5.08–5.64), P3 (5.64–6.06), P4 (6.06–6.26), P5 (6.26–6.87), P6 (6.87–7.78), P7 (7.78–8.37), P8 (8.37–8.73), P9 (8.73–9.59), P10 (9.59–10.08), P11 (10.08–10.16), P12 (10.16–10.49). Peptide amounts were: non-retained fraction (5.2 mg), P1 (15.6 mg), P2 (9.4 mg), P3 (3.5 mg), P4 (4.8 mg), P5 (5.4 mg), P6 (4.6 mg), P7 (3.9 mg), P8 (5.6 mg), P9 (4.7 mg), P10 (2.3 mg), P11 (4.7 mg), P12 (10.8 mg). The yield of the process (75.3 mg) was 90% in relation to

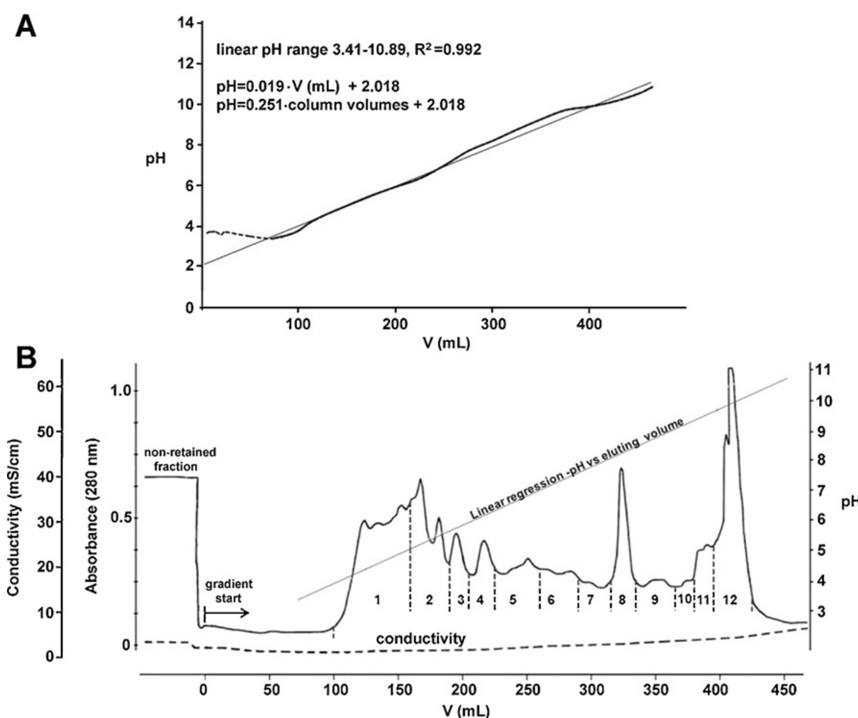

Fig. 2. (A) Generation of the linear pH gradient on the strong cation exchanger Fractogel EMD SO_3^- 650 M, from BM3 (250 mL) to BM12 (250 mL) at 1 mL/min, for the fractionation of the 1–10 kDa polypeptides from the *P. crucifer* secretion. (B) Corresponding ion-exchange chromatographic profile. One hundred fractions of 5 mL each were collected and grouped into 12 pools (P1–P12) marked on the chromatogram.

the expected 83.8 mg of retained peptides. A large non-retained fraction was collected, containing very acidic peptides (pI < 4) as well as low molecular weight compounds (with elution volumes between 1550 and 1660 mL in Sephadex G-50) that strongly absorbed at 280 nm and were not retained by the cation exchanger.

Subsequently, the peptides contained in P1–P12 were separated according to hydrophobicity differences, by using RPC18-HPLC. All fractions were collected, those absorbing at 280 nm were found within the range from 12 to 40 min. A total of 242 reversed-phase chromatographic fractions were analyzed by MALDI–TOF MS. Fig. 3A illustrates all reversed-phase chromatographic profiles; the most abundant fractions are signalized with their corresponding molecular masses. Two mass spectra, from abundant fractions P4–31 and P8–28, were selected as examples in Fig. 3B.

3.2. Peptide fingerprint

The use of reflectron mode in MALDI–TOF increases mass resolution at the expense of sensitivity, and introduces a mass range limitation [48–51]. Moreover, it exists the possibility of observing fragmentation ions using the reflector mode and mistaking these for contaminating peptide ions. Due to technical problems with respect to reproducibility in reflector mode, and given that the achieved mass accuracy was satisfactory for our application we used the instrument in linear mode.

MALDI–TOF measurements of the peptide bank from RP-HPLC generated a crude m/z data of more than 800 signals. A standard error of 0.1% was permitted and m/z values outside the error range were considered as different molecular masses. Closely related masses were averaged, when they appeared in adjacent reversed-phase fractions derived from the same or adjacent pools (P1–P12) of ion-exchange chromatographic fractions. Doubled charged species, ion adducts (+22, +38) and putative oxidized forms of the same molecule (+16, +32), were removed from the final data.

The MS analysis of the 242 reversed-phase chromatographic fractions yielded a peptide mass fingerprint composed of 504 different molecular masses (Tables 1 and 2, see Supplementary material), showing the wide peptide diversity present in *P. crucifer*, distributed within the molecular masses 1.1–9.8 kDa, comprising acidic and basic peptides eluted over the pH range 4.00–10.49 in the pH gradient IEC separation, and within 5–32% acetonitrile in RPC18-HPLC. The complete molecular mass and % acetonitrile data is shown in Table 1. Moreover, a simplified list organized in increasing order of molecular masses, is shown in Table 2 to facilitate quick searches of molecular mass values of interest.

Several features of the peptide composition of the *P. crucifer* secretion, derived from the chromatographic and mass spectrometry data, are summarized below:

- Main peptide biomarkers of *P. crucifer* secretion
  High intensity peaks from RP-HPLC with dominant mass signals are shown in Table 1 and Fig. 3A. The molecular masses of these abundant peptides from acidic pools (P1–P5) were: 2596, 5179, 5198, 6516, 6534, 6615 and 6746, and from basic pools (P6–P12): 2706, 2834, 3270, 3478, 3783, 4332, 4360, 4455, 4575, 4611, 5296, 5673, 6180, 6433, 6449, 6547 and 6871. These molecules were selected as biomarkers of the *P. crucifer* secretion. Two mass spectra of these biomarkers, P4–31 (6746 Da) and P8–28 (5296 Da), are shown as examples in Fig. 3B.
- Molecular mass vs. frequency
  The largest group comprised the smallest peptides, mainly within 2.0–3.5 kDa (Fig. 4A). These peptides were found in all pools of IEC fractions (Fig. 4B) and eluted in a wide range of concentration of acetonitrile (Fig. 4C), showing their diversity of isoelectric points and hydrophobicities.
- Molecular mass vs. elution pH

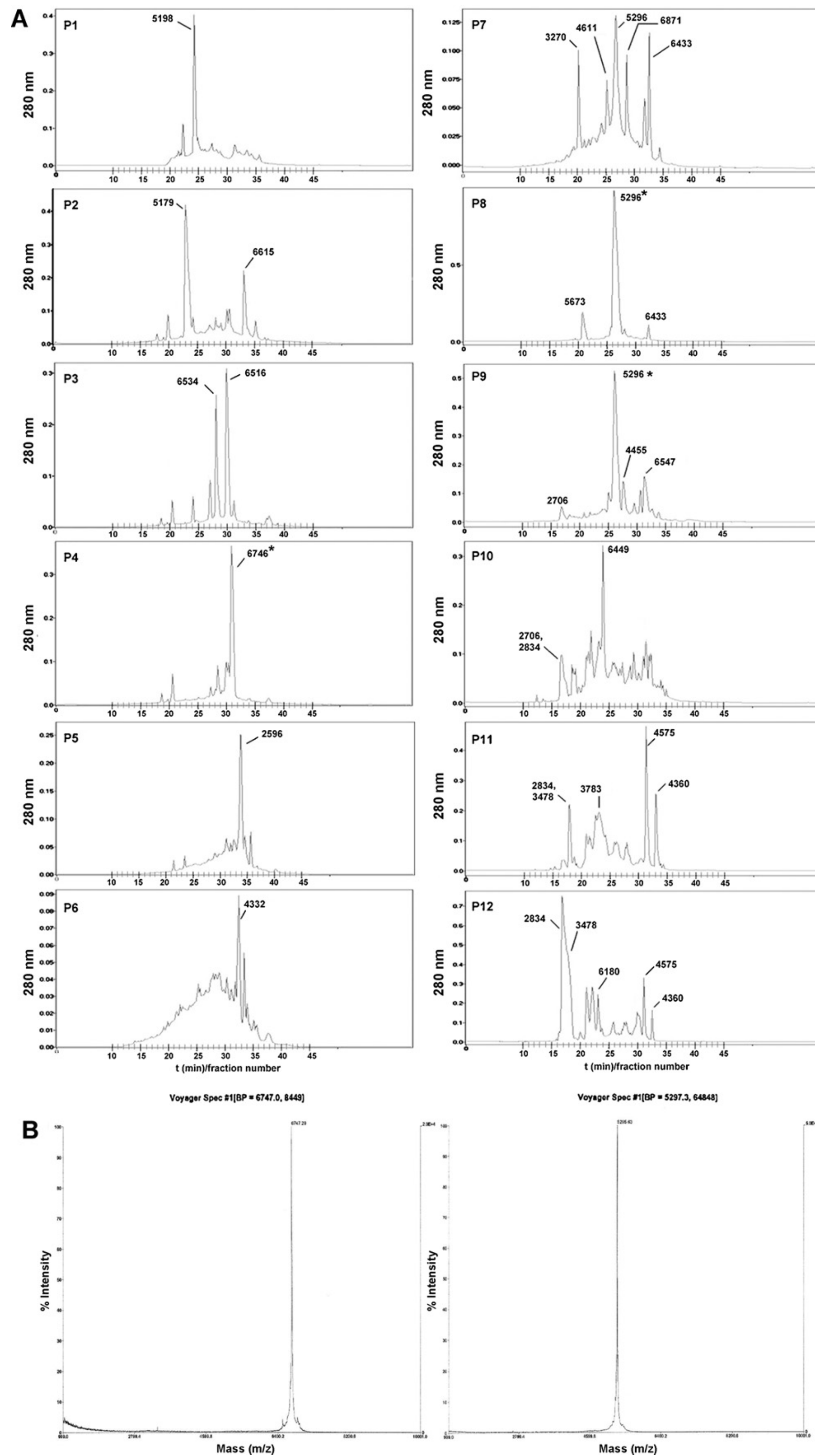

Fig. 3. (A) Reversed-phase C18 profiles of every pool (P1–P12) of IEC fractions obtained by pH gradient ion-exchange chromatography. Flow rate 2.5 mL/min; gradient from 2 to 52% acetonitrile in 50 min, then 52–95% acetonitrile in 5 min; detection wavelength 280 nm. Elution time (min) and fraction number are indicated on the horizontal axis. High intensity peaks with dominant mass signals are signalized with its corresponding molecular mass value. Two main fractions (P4–31 and P8–28), selected as examples, are marked with asterisk and their respective mass spectra are shown below. (B) MALDI–TOF spectra of two high intensity fractions, P4–31 and P8–28, with molecular masses of 6746 Da and 5296 Da, respectively. These peptides are among the main peptide biomarkers of the *P. crucifer* secretion.

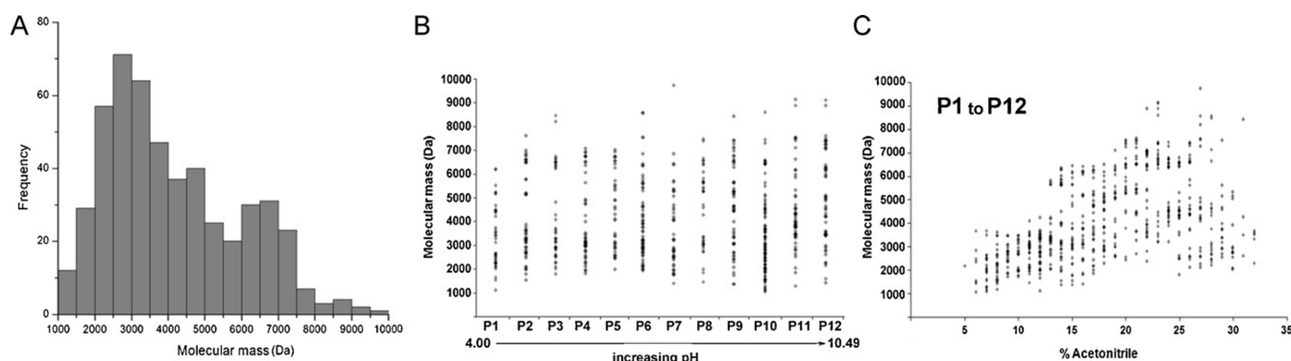

Fig. 4. Histogram and peptide maps obtained from the chromatographic and molecular mass data of *P. crucifer* secretion. (A) Histogram of molecular mass distribution of the 1–10 kDa peptides. (B) Molecular masses of peptides found in pools of IEC fractions (P1–P12) organized in order of increasing pH. (C) Molecular mass vs. % acetonitrile of eluted peptides from reversed-phase HPLC (increasing hydrophobicity). Darker dots in (B) and (C) indicate a higher number of dot overlaps, meaning peptides with close molecular masses and isoelectric points (B) and peptides with close molecular masses and hydrophobicity (C), respectively.

Every pool of IEC fractions contained peptides within a wide molecular mass range. No relationship between molecular mass and elution pH was found (Fig. 4B).

- Molecular masses vs. %ACN

In general, the most hydrophilic fractions contained peptides below 4 kDa, eluted at less than 15%ACN (Fig. 4C). Larger polypeptides started eluting at higher %ACN. The finding of higher molecular masses as the acetonitrile concentration gradient increases has been observed in previous animal venom studies [21,52–56]. By contrast, the smallest peptides eluted within the whole range of percentage of acetonitrile.

4. Discussion

A long history of isolation and characterization of sea anemone toxins has shown that molecules in the 1–10 kDa range are proteinaceous compounds, many of them exhibiting neurotoxic, protease inhibitor and cytolytic activities [6,12]. On the other hand, non-proteinaceous compounds have been found, such as purines, quaternary ammonium compounds, biogenic amines and betaines [57–59] but these are much smaller, less charged and less hydrophobic than peptides and proteins so they can be easily distinguished and separated in early purification steps.

The number of sea anemone species (39 up to date [60]) from which peptide and protein toxins have been isolated or discovered by molecular biology techniques represents a small fraction of the total known valid species, 1092 (Fautin, Daphne G. 2011. Hexacorallians of the World (http://geoportal.kgs.ku.edu/hexacoral/anemone2/index.cfm), indicating that the peptide diversity present in sea anemones remains mostly unexplored.

In recent years, genomic/transcriptomic and proteomic/peptidomic studies of sea anemones venoms have allowed the isolation and sequencing of new toxins belonging either to unclassified groups or known classes, from the species *N. vectensis* [14,15,18,19], *Bunodosoma cangicum* [21], *A. viridis* [16,20], *B. granulifera* and *S. helianthus* [17], demonstrating that sea anemones are more complex than estimated by any previous study based on bioassay-guided isolation of peptide toxins. However, the number of these studies remains very limited. To date, few peptidomic studies of sea anemones have been performed, involving only three species [17,21]. Moreover the separation strategy employed in these previous studies partially exploited the separation capacity of multidimensional chromatography, therefore the peptide diversities present in these sea anemone species may have been underestimated.

4.1. The first study of a sea anemone species from the family Phymanthidae

In the present work the 1–10 kDa peptide pool from the secretion of the unexplored sea anemone *P. crucifer* was fractionated by pH gradient ion-exchange chromatography and reversed-phase high performance liquid chromatography (RP-HPLC). The resulting samples were analyzed by mass spectrometry, generating venom peptide maps that describe the overall composition of the sea anemone secretion. Further experiments involving peptidome sequencing were not performed since this is beyond the purpose of the present work, which was only focused on a multidimensional separation strategy combined with molecular mass measurements for improving peptide fingerprinting of sea anemone secretion, starting from an unexplored species. Consequently, chemical characterization experiments were not required.

Our work represents the first study of the peptide fingerprint of a sea anemone species belonging to the family Phymanthidae, in contrast to families Actiniidae and Stichodactylidae, from which most known sea anemone peptide toxins have been isolated [6]. We found that the main peptide biomarkers of *P. crucifer* constitute a heterogeneous group of acidic and basic peptides having molecular masses from 2596 to 6871 Da. This result differs from those obtained in previous peptidomic studies of sea anemone species belonging to other families [17,21]. For example, very distinguishable 4–5 kDa peptide biomarkers were found in *B. granulifera* and *Bunodosoma cangicum* (genus Bunodosoma, family Actiniidae), mainly comprising APETx-like peptides and type 1 sodium channel toxins [17,21,61]. Main peptide biomarkers of *S. helianthus* (genus Stichodactyla, family Stichodactylidae) were found within the 3–4 kDa mass range, and did not include APETx-like peptides. In family Actiniidae, APETx-like peptides were originally isolated from *Anthopleura elegantissima* (APETx1 and 2). However, curiously, APETx-like peptides are completely absent in *A. viridis* (other species from family Actiniidae), when the near 40,000 ESTs publicly available are mined [20]. This suggests that the presence of certain classes of toxins may differ not only among different families but among different genera of the same family.

4.2. The number of secreted sea anemone peptides is larger than predicted. Our study vs. previous ones

The MALDI–TOF analysis of the reversed-phase fractions yielded 504 components, within the 1–10 kDa range, from the *P. crucifer* secretion. This number may represent an overestimation of the peptide components due to the putative presence of mass data redundancy and body parts contaminants in the sea anemone

secretion (see explanation below). On the other hand, the most acidic peptides were excluded from the present study so regardless the accuracy of the number of peptide components this is clearly an underestimate. Nonetheless we show that the number of peptide components (504) found in our work is much larger than in any other fingerprinting study of sea anemones, being highly superior to the previous estimation in *B. granulifera* (156 peptides), *S. helianthus* (113 peptides) [17] using the same extraction protocol as described in Section 2, and *B. cangicum* (81 peptides) [21], using an extraction protocol based on electric stimulation [62]. Our results indicate that the peptide composition of sea anemone secretion is dramatically more complex than predicted by previous studies. Several reasons account for this huge difference between *P. crucifer* and the other studied sea anemone species [17,21]. These are explained as follows:

- Enhanced peptide separation and detection.

  Previous peptidomic works on sea anemones employed a two step chromatographic separation methodology comprising gel filtration and reversed-phase chromatography [17,21]. In the present study, the separation capacity was enhanced by our multidimensional approach that included pH gradient IEC, between gel filtration and reversed-phase chromatography. We found no relationship between elution pH and molecular mass or hydrophobicity (elution %ACN), given that every pool of IEC fractions contained peptides spanning wide ranges of molecular mass (Fig. 4B) and hydrophobicity (Fig. 3A). Therefore pH gradient IEC is a suitable orthogonal step to be included in venom peptidomics of sea anemones or other venomous animals.

  pH gradient IEC combined with reversed-phase chromatography greatly improved the separation of peptides by fractionating them into several acidic and basic pools (P1–P12), which subsequently yielded a much greater number of reversed-phase chromatographic fractions (242 fractions from *P. crucifer*) in comparison with similar previous studies of other sea anemones (53 fractions from *B. granulifera*, 41 fractions from *B. cangicum* and 36 fractions from *S. helianthus*). A better separation reduces the number of coeluting peptides, which compete with each other for ionization and diminish detection in MS analyses due to ion suppression [48]. This is especially relevant to the analysis of low-abundance peptides, which usually escape detection due to the presence of high-abundance peptides [63]. Therefore, it is inferable that less ionizable peptides and many low-abundance peptides could be detected in the present study after improving resolution by combining pH gradient IEC and RP-HPLC.

  Moreover, the number of peptides detected was also increased by exploiting the high sensitivity of MALDI–TOF-MS in the analysis of fractions containing low peptide amounts. The off-line mass spectrometry analyses of previous peptidomic studies of sea anemones [17,21] were mainly focused on visible peaks manually collected according to UV-detection at 214 nm. However, low intensity peaks, valleys between peaks and flat zones in a chromatogram also contain peptides, which are likely to elute unnoticed so they might not be collected for MS analyses. This is critical in animal venom fingerprinting because many low-abundance peptides that exhibit strong biological activities at nanomolar (or even at subnanomolar) concentrations may remain undiscovered. In the present work a fraction collector was employed so a large range of fractions was collected and selected for molecular mass measurement, including many of them that apparently were not relevant (by UV-detection) but yielded high intensity peaks by MALDI–TOF analysis, such as P1–31, P1–36, P2–18, P2–19, P3–20, P3–23, P3–33, P3–37, P4–22, P4–23, P5–37, P5–38, P6–22, P6–23, P8–19, P8–23, P9–20, P9–36, P9–38, P10–15, P10–36, P11–15, P12–16.

- Wider molecular mass range

  Previous peptidomic studies of sea anemones [17,21] were focused on the 2–5 kDa peptide pool from Sephadex G-50. Sea anemone polypeptides with molecular masses outside these limits are likely to be less represented such as protease inhibitors, some potassium channel toxins [6], cytolytic peptides [6,12] and unclassified small paralyzing toxins [17]. Consequently, a wider mass range (1–10 kDa), previously analyzed in other venomous animals [28,64,65] was considered in the present work, aiming to include these families into the fraction of interest. This allowed us to find a notable population of 101 polypeptides (20% of the 1–10 kDa fraction) with molecular masses above 6 kDa in *P. crucifer*, in contrast to only 13, 3 and 4 from *S. helianthus*, *B. cangicum* and *B. granulifera*, respectively, representing 11.5%, 3.7% and 2.6% of the total number of peptides found in the neurotoxic fractions, according to the previous peptide fingerprint studies of sea anemones secretions [17,21].

- Peptide composition differences between sea anemone species

  In our previous study we compared (in identical conditions) two sea anemones species from different families [17], resulting in wide differences of peptide diversity according to: the reversed-phase profiles as well as the total number of peptides and crab-paralyzing toxins. *P. crucifer* belongs to a different family and this could be reflected on its peptide diversity. However the present study employed a methodology differing from previous ones, aiming to detect a larger number of peptides. Therefore we cannot assure that the peptide diversity present in *P. crucifer* is more complex than in *B. granulifera, B. cangicum* or *S. helianthus*. A study of these sea anemone species using the present methodology will probably reveal a larger number of peptides than previously reported [17,21].

- Molecular mass data redundancy

  Peptidomic and bioassay-guided studies of other venomous animals have provided information on several sources of redundancy in fingerprint data, which leads to overestimated numbers of venom peptides. Degradation or cleavage of proteins, missed cleavage sites from precursors and isoforms, non-specific post-translational modifications, isomasses and external contamination have been described [66] or some of them mentioned [30,31]. In the present study, commonly known sources of redundancy were removed from data, such as Na^+ and K^+ ion adducts, double charged species as well as redundant close mass values that were averaged when appeared in adjacent fractions. Moreover, the mass values of putative oxidized forms of the same molecule were removed, given that oxidation of methionine may be expected from the manipulation of the toxin [36]. However, sea anemone venoms have been much less studied than other animal venoms; therefore, additional molecular events that may lead to mass data redundancy cannot be discarded and should be investigated.

- Contaminants

  Lastly, we should not exclude the presence of some peptide contaminants from other parts of the body due to the extraction method (immersion in distilled water), which yields a clear salt-free sample but should be more aggressive and less selective than the extraction using electrical stimulation in marine isolated environment [62].

4.3. Unifying strategies for the analyses of sea anemone secretion

The comparison between venom peptidomic studies becomes difficult when different methodologies are employed, therefore a common methodology would beneficial for comparing peptide diversities among sea anemone species. However this may be a difficult task since criteria and technologies are variable among

research groups. Nevertheless some general advices should be helpful to make data more comparable from one study to another.

A) The mass range covered by the study should be always the same; 1–10 kDa is more suitable mass range than 2–5 kDa. In our study we collected all fractions within the complete peptidomic range of 1–10 kDa to include peptides of interest with molecular masses outside the 2–5 kDa range previously defined as the neurotoxic fraction [27] and analyzed in peptidomic approaches [17,21] of sea anemones. Currently, the separation of the peptide pool from the sea anemone secretion has been done by gel filtration in Sephadex G-50. Nonetheless, other gel filtration media (FPLC and HPLC) are suitable such as Superdex Peptide [67] and Superdex 30 [68–70], which provide faster separations, higher resolution and appropriate selectivity for fractionating the 1–10 kDa peptides. The column can be calibrated by using molecular weight standards as described by Lagos [27] (a similar procedure was used in the present work) or by SDS-PAGE analysis of eluting fractions [28].
B) The use of multidimensional approaches including ion-exchange chromatography. The use of reversed-phase C18 HPLC after gel filtration chromatography has proven to be a fast and effective separation strategy for bioassay-guided isolation [17,21,71–76] and peptidomic analysis of sea anemones [17,21]. However the use of reversed-phase chromatography may not be sufficient to approach the fractionation of a complex peptide population containing many low-abundance peptides, especially when they co-elute with interfering high intensity fractions. Therefore another chromatographic dimension should be included into the separation strategy for more suitable fractionations of such as complex samples. Ion-exchange chromatography is an orthogonal high resolution technique, with high loading capacity that fractionates and concentrates peptides, allowing the enrichment of minor components, especially very basic peptide toxins from sea anemones, such as potassium channel toxins [36,37,41,42,77] and an acid-sensing ion channel toxin [77]. Therefore including this separation principle improves separation, reduces sample complexity and concentrates peptides prior to the reversed-phase chromatographic step, facilitating subsequent detection by mass spectrometry. In IEC, protein mixtures can be fractionated by either salt gradient or pH gradient elution [78]. Therefore a question arises: salt gradient IEC or pH gradient IEC? Salt gradient IEC is widely employed in protein purification due to its ease of use, loading capacity, versatility and high resolution. However, proteins with the same number of effective charges are released from the ion exchanger close to each other, thus limiting the selectivity of salt gradient IEC when complex mixtures are separated [79]. On the other hand, pH gradient IEC is a powerful technique for the separation of proteins having close charge properties; therefore pH gradient has been usually employed for separating proteins with close isoelectric point (pI) values [78]. In pH gradient IEC proteins usually elute in order of their pI [78–80], and this order is frequently not preserved using salt gradient IEC, thus pH gradient IEC should be more suitable than salt gradient IEC for separating distinct structural classes of toxins having isoelectric point differences. Moreover pH gradient fractionation can be used as an analytical tool for design and pH optimization of salt gradient ion-exchange chromatography; and both modes can be combined for fractionation of crude samples [78,81]. Therefore, selecting one or both IEC modalities will require preliminary experimental evaluation for better exploitation of IEC. Lastly, the use of ion-exchange high resolution media (FPLC and HPLC columns) is recommendable as they provide better and faster separations than low pressure media, thus reducing sample complexity and ion suppression events in MS analysis. High resolution IEC media produce a higher number of IEC fractions with different peptide composition; this is more effective than simply dividing a low resolution chromatographic profile into more fractions, which leads to an increase of the number of analyses, time consumed as well as more data redundancy given the presence of the same peptides in adjacent IEC fractions.
C) Maximizing peptide recovery for detection by mass spectrometry: on-line and off-line analyses. On-line multidimensional chromatography minimizes losses because of the continuous monitoring provided by LC–LC coupling through controlled valves; moreover, its fully automated performance allows fast analyses. On the other hand, off-line multidimensional chromatography is easy to carry out by collection of column effluent; moreover it concentrates trace solutes from large volumes and can work with two LC modes that use incompatible solvents [82]. Off-line multidimensional chromatography is time consuming and caution must be taken to avoid losses or contamination due to handling of samples between chromatographic steps. Nonetheless, off-line LC–LC [28,83,29,30,64] are used more extensively than on-line LC–LC [31] for animal venoms analyses, probably due to its flexibility for optimization and ease of use, which does not require fully automated equipment.

Likewise, on-line and off-line LC–MS approaches are used for venom peptide analyses. On-line LC–MS, yields sensitive, accurate and automated analyses, mainly performed by on-line LC–ESI-MS, although on-line LC–MALDI-MS instruments are also available [84]. On the other hand, the off-line approaches allow LC and MS to function and operate as two independent systems, which may be individually optimized [85]. If an off-line procedure will be used, a fraction collector is recommendable to avoid the reliance of manual collection on detection methods having less sensitivity than mass spectrometry techniques, such as UV-detection, currently used in LC systems for the detection of peptides and proteins. The off-line coupling (including fraction collection) of MALDI–MS to liquid chromatography and capillary electrophoresis has been reviewed [86].

Several MS equipments and LC–MS combinations have been employed for venom peptide fingerprinting, including on-line ESI-MS [87,88], off-line ESI-MS [21,31] and off-line MALDI–MS [17,21,56,89] analyses as well as several other studies employing several approaches for comparison among them [30,66,90,91]. Newton et al. [30] showed that MALDI–TOF-MS detected more peaks at m/z value > 5000 than on-line LC–ESI-MS. Pimenta et al. [66] used an off-line process that allowed the identification of more molecular species than both direct MALDI–TOF-MS detection and on-line LC–ESI-MS analyses. Legros et al. [90] showed that on-line LC–ESI-MS, MALDI–TOF-MS and nanoESIMS are complementary methods, while nanoESI-QqTOFMS is the most sensitive and the fastest technique, to draw up a complete map of the mixture components. On the other hand, Songping [28] indicated that in proteome profiling of spider venoms good results are achievable with any of the following instruments MALDI–TOF-MS, MALDI–TOF/TOF-MS/MS, ESI-Q–TOF-MS/MS, ESI-iTRAP-MS/MS and ESI-FT-ICR MS/MS, but these ones and the protocols require careful optimizations and skilful interpretation of data.

Thus, there is a variety of on-line and off-line LC–LC and LC–MS combinations that can be used for animal venom analyses. On-line approaches provide fast and automated analyses that allow covering a large number of fractions. Off-line approaches are more flexible thus allowing optimization of separation and detection. In any case using off-line analyses (LC–LC or LC–MS) for venom peptide fingerprinting, automated fraction collection is recommendable. Moreover it should be remembered that peptide detection prior to mass spectrometry analysis is a complement. The

final detection should rely on the high sensitivity and resolution provided by mass spectrometry in order to obtain a more complete overall picture of the venom peptides.

4.4. Buffer systems for pH gradient IEC and the present study

Customarily, pH gradient IEC has been much less used than salt gradient IEC because linear pH gradients cannot be obtained simply by mixing buffers of different pH in linear volume ratios since the buffering capacities of the systems produced are pH dependent [92]; moreover ion strength also changes thus influencing the retention of proteins and peptides. Therefore, pH gradient are generally used over narrow pH intervals (max. 2 pH units) using the same buffer system at two different pH values. Nonetheless, several studies [79,93–98] have successfully achieved the formation of wide linear pH gradient either on weak or strong ion exchangers, using a combination of simple buffers (e.g. pISep, composed of piperazine, *N*-methyl piperazine, bis–tris propane and triethanolamine) or complex mixtures (e.g. chromatofocusing polybuffers), resulting in high resolution separations of protein and peptides according to isoelectric point differences. However pH gradient IEC has not been used in peptidomic studies of sea anemones [17,21] or other venomous animals [28–31,64,83]. Our work introduced pH gradient IEC into venom peptidomics by using our own buffer mixture (BMpH), which similarly to several known buffers, is easy to prepare and produces a linear pH gradient over a wide pH range at low ion strength. Nonetheless, additional studies should be done by using other ion exchangers, different pH gradients and a variety of complex samples in order to evaluate its versatility for other applications.

5. Conclusions

The separation and detection of a large number of peptides present in complex mixtures, such as animal venoms, can be improved by using multidimensional separation strategies comprising orthogonal steps, which increase resolution and detection of components. Considering the known powerful separation capacity of multidimensional chromatography we introduced pH gradient IEC into venom peptidomics, which combined with reversed-phase chromatography and mass spectrometry revealed that sea anemone secretion is much more complex than previously estimated. Therefore a wider scope for finding novel biologically active compounds should be expected from these organisms. Further extensive work comprising the chemical and pharmacological characterization of the secretion peptidome will allow the finding of new members of known classes of toxins as well as other peptides to be classified into new classes.

Conflicts of interests statement

The authors declare that there are no conflicts of interests.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.06.034.

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Glossary

t_D: system dwell time (min)
Φ: volume fraction of B solvent in the mobile phase
t_G: gradient time (min)
t_M: column dead time (min), retention time of an unretained peak
t_R: retention time (min)
t_{delay}: gradient delay time (min), corresponding to initial isocratic elution before the start of the gradient
V_R: retention volume (mL)
M_r: relative molecular mass