SEPARATION AND IDENTIFICATION OF PEPTIDES IN HYDROLYSED PROTEIN EXTRACTS FROM EDIBLE MACROALGAE BY HPLC-ESI-QTOF/MS

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ABSTRACT

Macroalgae contain significant amounts of high-quality proteins which, because of their structural diversity, contain a range of yet undiscovered peptides within their primary structures. In this work, an analytical methodology was developed for the separation and identification of peptides present in protein hydrolysates from three different edible macroalgae used for human consumption (*Saccharina latissima* (brown macroalga), *Codium spp*. (green macroalga), and *Mastocarpus stellatus* (red macroalga)). The extraction of aqueous and alkaline soluble proteins was carried out followed by their precipitation with HCl or acetone. The protein extracts obtained were submitted to enzymatic digestion with alcalase and subsequently analyzed by reversed-phase high-performance liquid chromatography-quadrupole-time-of flight mass spectrometry (RP-HPLC-QTOF/MS) and *de novo* sequencing tool to separate and identify different short chain peptides. Thirty-seven peptides were identified in the hydrolysed protein extracts from the three macroalgae, five of them being common in brown and red macroalgae. After checking against BIOPEP database, several sequenced peptides were found within longer peptides with potential antibacterial activity. Any of the identified peptides had previously been identified in macroalgae.

Keywords: Peptides; reversed-phase; liquid chromatography-tandem mass spectrometry; macroalgae**.**

1. INTRODUCTION

Macroalgae are a diverse group of marine organisms which generate a wide group of functional biomolecules to survive under stress conditions [1]. They produce high-quality proteins whose concentrations can vary from 5 to 15% in the case of brown algae (Phaeophyta), from 9 to 26% for green algae (Chlorophyta) and from 10 to 47% for red algae (Rhodophyta) (percentages referred to dry weight) [2]. Peptides contained in proteins from marine sources, which can be released during enzymatic hydrolysis, food processing or ripening [3], have a high interest since they could present different type of bioactivity such as anti-cancerous, anti-proliferative, anti-coagulant, antibacterial, antifungal, and anti-tumor, among others [4-9]. Although peptides contained in protein hydrolysates from macroalgae could present some type of bioactivity, their separation and identification in these macroalgae protein hydrolysates have scarcely been investigated [10-15].

One of the most relevant challenges to obtain peptides from macroalgae is related to the extraction of proteins from the matrix since it is a topic which has not been studied deeper compared to the extraction of proteins from crops [16, 17] Protein extraction from macroalgae is a difficult task due to the cross-linking between polysaccharides and proteins within the matrix, as well as the inaccessibility of proteins within macromolecular cell wall assemblies [18]. The cross-linking between polysaccharides and proteins is especially important for brown macroalgae [19]. For instance, the extraction of proteins from the Laminaria alga *Saccharina japonica* has proved to be difficult due to the high levels of non-protein compounds (mainly viscous polysaccharides) whose presence interferes with protein extraction [20]. As a consequence, the main methods used for the extraction and precipitation of proteins are not completely useful in macroalgae.

High-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) is the analytical technique mainly employed to carry out the separation and identification of peptides [21]. Nowadays, most of the MS systems are able to detect with accuracy peptides with a length higher than 5 amino acids. However, the analysis of shorter peptides with 2 to 4 amino acids has scarcely been reported in the literature [21]. For instance, the low or high fragmentation of short peptides by tandem MS can make their detection difficult and challenging [21-23]. Thus, the development of analytical methods to carry out the separation and identification of short chain peptides presents a high interest when an in deep characterization of food is attempted.

The aim of this work was to separate and identify peptides contained in protein hydrolysates from three different edible macroalgae (*Saccharina latissima*, *Codium spp*. and *Mastocarpus stellatus*) used for human consumption. The extraction of aqueous and alkaline soluble proteins was carried out followed by protein precipitation using different approaches. Protein extracts obtained were subsequently submitted to enzymatic digestion and analyzed by reversed phase high-performance liquid chromatography coupled to a quadrupole-time-of flight mass spectrometer (RP-HPLC-QTOF/MS) and *de novo* sequencing tool.

2. MATERIALS AND METHODS

2.1 Chemicals and samples

All chemicals and reagents were of analytical grade. Sodium hydroxide, bovine serum albumin (BSA), and thermolysin were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid, acetone, methanol, ethanol and acetic acid were acquired in Scharlau (Barcelona, Spain). Sodium dodecyl sulfate (SDS) was purchased from Merck (Darmstadt, Germany). Alcalase 2.4 L FG was kindly donated by Novozymes

Spain S.A. (Madrid, Spain). Mini-protean precast gels, Laemmli buffer, Tris/glycine/SDS running buffer, precision plus protein standards (recombinant proteins expressed by Escherichia coli with molecular weights of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa), silver stain kit, and Bradford reagent (Coomassie Blue G-250) were acquired from Bio-Rad (Hercules, CA, USA).

For the HPLC-MS/MS analysis, MS grade methanol and formic acid from Sigma-Aldrich were employed. The ultrapure water used was obtained from a Milli-Q (Millipore, Bedford, MA, USA) instrument.

Macroalgae samples (*Saccharina latissima*, *Codium spp*. and *Mastocarpus stellatus*) consisted in dried algae kindly donated by Porto-Muíños, S.L. (La Coruña, Spain). Once the macroalgae were collected, they were washed, dried at 30-35 ºC and grinded.

2.2 Total protein content

The protein content of the macroalgae was determined by the Kjeldahl method [24]. Nitrogen data were converted into protein values employing a conversion factor of 6.25 and were expressed as g per 100 g of dried macroalga. Analyses were performed in triplicate.

2.3 Extraction of proteins

The procedure used for the extraction of water and alkaline soluble proteins from milled dried macroalgae was based on the method described by Harnedy and FitzGerald (2015) [18] with some modifications (see Figure 1). In brief, 0.5 g of dried milled macroalgae powder was suspended in milli-Q water (1:20 (w/v)) and stirred gently for 3 h at 4 ºC. The proteins in the aqueous extract were removed by centrifugation at 4000 x g for 15 min at 4 ºC.

For alkaline soluble protein extraction, the pellet obtained after centrifugation was resuspended in 0.12 M NaOH at a weight volume ratio of 1:15 (w/v) and stirred gently at

room temperature for 1 h. Alkaline extraction was performed twice and both supernatants obtained by centrifugation at 4000 x g for 15 min at room temperature were combined. Then, proteins from the aqueous and alkaline extracts were precipitated employing two different methods. First, the proteins were precipitated by adjusting the pH of each extract to around pH 3.5 using HCl. Aqueous protein extracts were kept for 30 min at 4 ºC while alkaline protein extracts were kept at room temperature to achieve the precipitation. The solutions were centrifuged at 4000 x g for 15 min at 4 ºC, and the pellets obtained were collected and dried by vacuum-drying. The proteins obtained were called aqueous or alkaline proteins precipitated with HCl (WPHCl and APHCl, respectively).

Secondly, the remaining proteins in both supernatants, aqueous and alkaline solutions after protein precipitation with HCl, were subjected to a second precipitation using cold acetone. The supernatants were diluted twice their volume in cold acetone and allowed then to stand for 1 h at -8 ºC. The solutions were centrifuged at 4000 x g for 15 min at 4 ºC, and the pellets obtained were over-night dried at room temperature. The proteins obtained were called aqueous or alkaline proteins precipitated with acetone (WPA and APA, respectively). The protein content for both aqueous and alkali extracts precipitated with HCl and acetone was estimated by Bradford assay [25]. WPHCl, APHCl, WPA and APA were ready to be subjected to protein digestion. Protein extraction for each algae was carried out in triplicate.

2.4 SDS-PAGE

Proteins were separated by SDS-PAGE using a Bio-Rad Mini-protean system (Hercules, CA, USA). Proteins solutions were mixed with Laemmli buffer containing 5 % (v/v) β-mercaptoethanol, followed by heating at 100 °C during 5 min and loaded into commercial Mini-PROTEAN TGX Precast Protein Gels from Bio-Rad (Hercules, CA, USA). Proteins were separated by applying 80 V for 5 min and 150 V until the separation was completed

using Tris/glycine/SDS as running buffer. Molecular markers of standard proteins with molecular weights from 10 to 250 kDa were also run. After separation, proteins were treated with a fixing solution of water/MeOH/acetic acid (50/40/10 % (v/v)) by shaking for 30 min and then with a second fixing solution water/EtOH/acetic acid (85/10/5 % (v/v)) twice for 15 min each. Gels were then treated with an oxidizer solution for 5 min and washed with water followed by the addition of the silver reagent and shaking during 20 min. Afterwards, the gel was washed for 1 min with water and developer solution was added. Reaction was stopped by adding 5 % acetic acid solution.

2.5 Protein digestion

Protein extracts obtained from macroalgae were hydrolyzed using the enzyme alcalase following a procedure previously optimized by our research team for the hydrolysis of proteins from plum by-products [26]. The protein extracts were dissolved in 5 mM borate buffer (pH = 8.5) at a final concentration of 5 mg/mL with the help of an ultrasonic probe for 5-10 min and with 30 % of wave amplitude. Then, the enzyme was added at an enzyme/substrate ratio of 0.15 AU/g protein and the solution was incubated in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany) at 50 ºC with agitation (700 rpm) for 4h. The digestion was stopped (100 ºC for 10 min) using Thermomixer Compact and the solution was centrifuged for 10 min at 6000 g. Finally, the supernatant was collected for its analysis by HPLC-ESI-MS/MS.

2.6 Separation and identification of peptides by RP-HPLC-ESI-QTOF/MS

Peptide analysis was performed using an HPLC system 1100 from Agilent (Agilent Technologies, Santa Clara, CA, USA) coupled to a quadrupole-time-of flight mass spectrometer (QTOF/MS) Agilent 6530 equipped with an orthogonal electrospray ionization (ESI) source (Agilent Jet Stream, AJS). The HPLC instrument was equipped with a quaternary solvent pump, an auto-sampler, and a column heater compartment.

Agilent Mass Hunter Workstation software B.07.00 from Agilent was employed for HPLC and MS control, data acquisition, and data analysis.

The separation was carried out using a porous-shell fused-core Ascentis Express C18 analytical column (150 mm x 2.1 mm, particle size 2.7 μm) with an Ascentis Express C18 guard column (0.5 cm × 2.1 mm, 2.7 μm particle size), both from Supelco (Bellefonte, Pa, USA). The column temperature was 50 ºC and the flow rate 300 μL/min. Five μL of extract were injected. The mobile phases consisted of (A) water with 0.5 % formic acid and (B) methanol with 0.5 % formic acid in a gradient elution analysis programmed as follows: 0 min, 1 % (B); 0-5 min, 1 % (B); 5-10 min, 1-5 % (B); 10-30 min, 5-60 % (B); 30-35 min, 60 % (B), with 15 min of post-time.

The mass spectrometer was operated in positive ion mode and the mass range was from 100 to 1700 m/z. MS parameters were the following: capillary voltage, 3500 V; nebulizer pressure, 50 psig; drying gas flow rate, 12 L/min; gas temperature, 350 ºC. The fragmentor voltage (cone voltage after capillary) was set at 80 V. The skimmer and octapole voltage were 60 V and 750 V, respectively. Source sheath gas temperature and flow were 400 ºC and 12 L/min, respectively. MS/MS was performed employing the auto mode and the following optimized conditions; 1 precursor per cycle, dynamic exclusion after two spectra (released after 1 min), and collision energy of 5 V for every 100 Da. Internal mass calibration of the instrument was carried out using an AJS ESI source with an automated calibrant delivery system. The reference compound solution for internal mass calibration containing purine and HP-0921 (hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine) in acetonitrile-water (90:10, v/v) (4 μM and 2.5 μM, respectively, 15 μL/min) from Agilent was used, *m/z* 121.0509 and *m/z* 922.0098, respectively. The analyses were conducted in triplicate.

Tandem MS/MS spectra were obtained for the molecular ion with the highest abundance. Every sample was injected in triplicate into the MS system. In order to assure that identified peptides came from macroalgae protein, MS/MS spectra were analyzed using PEAKS Studio Version 7 (Bioinformatics Solutions Inc., Waterloo, Canada). Data analysis was performed by *de novo* sequencing tool. Only those peptides identified with an ALC (expected percentage of correct amino acids in the peptide sequence) above 85% and with a good precursor fragmentation pattern were considered. Moreover, only those peptides appearing in at least 7 injections from 9 injections (three injections of each triplicate) were taken into account. Only isoforms with leucine (L) are presented in our results, although peptide sequences containing isoleucine (I) amino acid instead of L are also possible since it is not possible to differentiate I from L by the MS used.

3. RESULTS AND DISCUSSION

3.1. Development of an analytical methodology for the separation and identification of peptides by RP-HPLC-ESI-QTOF/MS

To achieve the separation and identification of peptides in hydrolysates from macroalgae protein extracts, an adequate analytical methodology, based on the use of HPLC-MS/MS, was developed. Taking into account that the protein contents for the three studied macroalgae (determined as described in section 2.1) were 6.3 ± 0.1 % for *Saccharina latissima*, 12.4 ± 0.8 % for *Codium spp*., and 16.9 ± 0.5 % for *Mastocarpus stellatus* (all percentages referred to sample dry weight), the macroalga *M. stellatus* was selected to perform the optimization of the chromatographic and MS parameters due to its higher protein content. Then, a protein aqueous extract was obtained and precipitated with HCl (WPHCl) following the protocol previously described (see section 2.3). The protein extract obtained was hydrolyzed and analyzed by HPLC-MS/MS using a C18 column. To

optimize the separation conditions, the effect of different parameters such as gradient program (gradient time, gradient shape, and initial composition of the mobile phase), column temperature (25-50 °C), flow rate (0.2-0.4 mL/min), and injection volume (2-5 µL), was investigated. The best resolution and shortest analysis time were achieved using a gradient elution based on water with 0.5 % formic acid (solvent A) and methanol with 0.5 % formic acid (solvent B) programmed as follows: 0 min, 1% B; 0-5 min, 1% B; 5-10 min, 1-5% B; 10-30 min, 5-60 % B; 30-35 min, 60% B, with 15 min of post-time at final composition. The other selected experimental conditions were a flow rate of 0.3 mL/min, a column temperature of 50 °C, and an injection volume of 5 µL. MS/MS parameters for peptide identification were selected taking into account those previously employed to identify peptides from different sources such as food and food by-products [26-28]. MS/MS data obtained using the developed method were analyzed using the *de novo* sequencing tool from the PEAK Software.

Figure 2 shows the Total Ion Chromatogram (TIC) corresponding to the analysis of protein hydrolysates from *M. stellatus* and the mass spectrum showing the fragmentation pattern of peak at 21.9 min and 600.3320 m/z (VGGTGPL peptide). As it can be observed, a good chromatographic profile could be obtained in an analysis time of 35 min.

3.2. Protein extraction and digestion

Protein extracts were obtained following the protocol described by Harnedy and FitzGerald (2015) [18] with some modifications. The method involved two subsequent aqueous and alkaline extractions under the conditions described in section 2.3. Protein profiles obtained by SDS-PAGE were obtained and compared for the three algae showing electrophoretic profiles with bands at molecular mass values lower than 25 kDa for *M. stellatus* and intense bands corresponding to molecular mass between 75 and 250 kDa

(see **Figure S1 from supporting information**) for the other two macroalgae. The total protein content (expressed in %) obtained from four extracts (WPHCl, APHCl, WPA and APA) from each macroalgae, estimated by Bradford assay, was 1.8 ± 0.5 % for *Saccharina latissima*, 2.7 ± 0.5 % for *Codium spp*., and 4.7 ± 0.7 % for *Mastocarpus stellatus*. showing higher extraction yields for the red and green macroalgae since these macroalgae presented higher crude protein content before extraction. Protein extracts obtained were precipitated with HCl and digested with the enzyme alcalase. Under these conditions, 12 and 17 peptides were found in aqueous extracts (WPHCl) and 2 and 14 peptides in alkaline extracts (APHCl) from *S. latissimi* and *M. stellatus*, respectively. However, peptides were not obtained from both extracts from *Codium spp*. Moreover, a gel formation was observed in *S. latissima* after the precipitation of proteins with HCl. This fact could be explained by the high levels of non-digestible viscous polysaccharides that make especially problematic the extraction of proteins from brown macroalgae [20, 29].

Thus, based on the experience of our research group on the analysis of peptides from different sources, protein precipitation was carried out using cold acetone instead of HCl (see section 2.3) [30]. Under these conditions, 11, 6, and 11 peptides were obtained in aqueous extracts from *S. latissimi*, *Codium spp*., and *M. stellatus,* respectively, and 14 and 10 peptides in alkaline extracts of *S. latissimi* and *M. stellatus* (peptides were not found in the alkaline extract for *Codium spp)*. Bearing in mind the results obtained with HCl and acetone, a combination of both approaches was evaluated. Thus, aqueous and alkaline extracts were firstly precipitated with HCl by adjusting the pH to around 3.5 (extracts WPHCl and APHCl, respectively). Then, the supernatants obtained were submitted to a second precipitation by adding cold acetone (extracts WPA and APA, respectively) (see Figure 1). Following this procedure, 12, 2, 11 and 14 peptides were

obtained in WPHCl, APHCL, WPA and APA extracts, respectively, from *S. latissima*; 6 peptides were detected in WPA extracts while any peptide was not found in WPHCl, APHCL and APA extracts from *Codium spp*., and 17, 14, 11 and 10 peptides were got in WPHCl, APHCL, WPA and APA extracts from the macroalga *M. stellatus,* respectively (see Tables 1, 2 and 3). Since a higher number of peptides could be obtained following this approach, it was selected to carry out the isolation of proteins from the different macroalgae.

Although two different enzymes, alcalase and thermolysin, were tested for protein digestion under the experimental conditions previously employed by our research team [26, 31], alcalase was chosen to achieve the hydrolysis since most of the peptides obtained using thermolysin belong to the protein sequence of this enzyme.

3.3. Peptide identification in protein hydrolysates

In order to carry out the tentative identification of peptides in the hydrolyzed protein extracts from *S. latissima*, *Codium spp*., and *M. stellatus*, they were analyzed by the developed HPLC-MS/MS method. Then, MS/MS data were treated by the PEAKS software to obtain *de novo* sequence.

Figures 2, 3 and 4 show the TIC chromatograms corresponding to the protein hydrolysates from WPHCl extract in *M. stellatus,* APA extract in *S. latissima* and APA extract in *Codium spp.,* respectively. These selected hydrolysates extracts presented the highest number of peptides for each macroalga. Moreover, these figures also display as an example, the mass spectrum with the fragmentation pattern of VGGTGPL, LNVE and TSFLDL peptides, respectively.

Tables 1, 2 and 3 show the different peptides identified in *M. stellatus, S. latissima* and *Codium spp*., respectively, along with their experimental molecular masses, ALC, and

accuracy. Forty-nine different peptides with a number of amino acids ranging from 4 to 10 were identified. As it can be seen in Figure 5, the Venn diagram showed eleven common peptides in *S. latissima* and *M. stellatus* (ATLN, SLGGAS, LNVE, ATYLGS, APGAGVY, LNVEAA, SVGAELE, VLDTGLQ, VSLY, VAVL, and MGDVLNM) and non-common peptides with *Codium spp*. (see Figure 5a and Tables 1, 2 and 3). Twelve peptides found in *S. latissima* and *M. stellatus* (VAGAA, SVGAE, ATLN, YYGK, ASHPDLN, ATYLGS, SHPDLN, APGAGVY, SVGAELE, VSLY, VAVL, SVGAEL) could belong to the alcalase enzyme sequence. Thus, thirty-seven different peptides were found in the three macroalgae being five of them common in *S. latissima* and *M. stellatus* (SLGGAS, LNVE, LNVEAA, VLDTGLQ, and MGDVLNM) (see Figure 5b).

The data obtained demonstrated that the highest number of peptides were found in the red macroalga *M. stellatus* (25 peptides), followed by the brown (*S. latissima*) (11 peptides) and green (*Codium spp*.) (6 peptides) macroalgae. Moreover, to the best of our knowledge, this is the first time that these peptides have been found in these macroalgae. The amino acid composition of the identified peptides in these macroalgae had high percentage of hydrophobic amino acids (leucine (L)/isoleucine (I), proline (P) and valine (V)) within their sequences.

In order to know the potential bioactivity of the identified peptides found in these macroalgae, they were verified against BIOPEP database [32]. Several sequences of peptides were found within longer peptides with potential bioactivities (see Tables 1, 2 and 3). For instance, the peptides VLNE, VIAE, VTSL, VVGQ and LDLY were previously found within a longer sequence of antibacterial peptides. However, most of the peptides found in these macroalgae have not previously been reported.

4. CONCLUSIONS

An analytical methodology was developed for the first time enabling the separation and identification of short chain peptides from three edible macroalgae, *M. stellatus, S. latissima* and *Codium spp*. The extraction of aqueous and alkaline soluble proteins was achieved followed by their precipitation and enzymatic hydrolysis with alcalase enzyme. Peptide hydrolysates were analyzed by HPLC-MS/MS and *de novo* sequenced using PEAKS software. Thirty-seven peptides were identified in the three macroalgae, being five of them common in *M. stellatus* and *S. latissima*. The peptides identified in these samples were not previously found in macroalgae. After checking against BIOPEP database, several sequenced peptides were found within longer peptides with potential bioactivities mainly with antibacterial properties.

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Declaration of author contributions

All authors were involved in the conception and design of the study. R.P.M performed most experimental work and data acquisition. M.L.M. was responsible for getting financial support. All the authors contributed to data interpretation and the writing of the manuscript, performed its critical revision and approved the final manuscript.

Conflicts of interest

The authors have no conflict of interest to disclose.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable

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FIGURE CAPTIONS

Fig. 1 Protein extraction procedure employed in this work.

Fig. 2 Total ion chromatogram from WPHCl extracts hydrolysed with alcalase from *Mastocarpus stellatus* by RP-HPCL-ESI-QTOF-MS/MS and an example of MS/MS spectrum of the peptide VGGTGPL observed at 21.9 min (molecular mass (Da): 599.3279).

Fig. 3 Total ion chromatogram from APA extracts hydrolysed with alcalase from *Saccharina latissima* by RP-HPLC-ESI-QTOF-MS/MS and an example of MS/MS spectrum of the peptide LNVE observed at 18.8 min (molecular mass (Da): 473.2485).

Fig. 4 Total ion chromatogram from WPA extracts hydrolysed with alcalase from *Codium spp*. by RP-HPLC-ESI-QTOF-MS/MS and an example of MS/MS spectrum of the peptide TSFLDL observed at 30.9 min (molecular mass (Da): 694.3538).

Fig. 5 Venn diagram of peptides identified in *Mastocarpus stellatus, Saccharina latissima* and *Codium spp* taking into account the peptides in common with the alcalase enzyme protein sequence (a) and without taking into consideration the peptides in common with the alcalase enzyme protein sequence (b).

Figure 1.

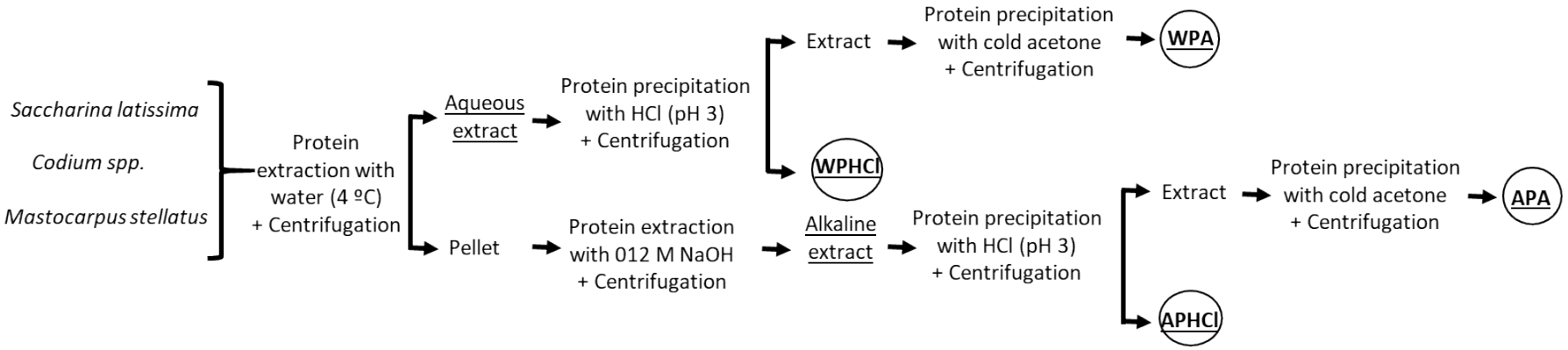

Saccharina latissima
Codium spp.
Mastocarpus stellatus
Protein extraction with water (4 ºC) + Centrifugation
Aqueous extract
Protein precipitation with HCl (pH 3) + Centrifugation
Extract
Protein precipitation with cold acetone + Centrifugation
WPA
WPHCl
Pellet
Protein extraction with 012 M NaOH + Centrifugation
Alkaline extract
Protein precipitation with HCl (pH 3) + Centrifugation
Extract
Protein precipitation with cold acetone + Centrifugation
APA
APHCl

Figure 2.

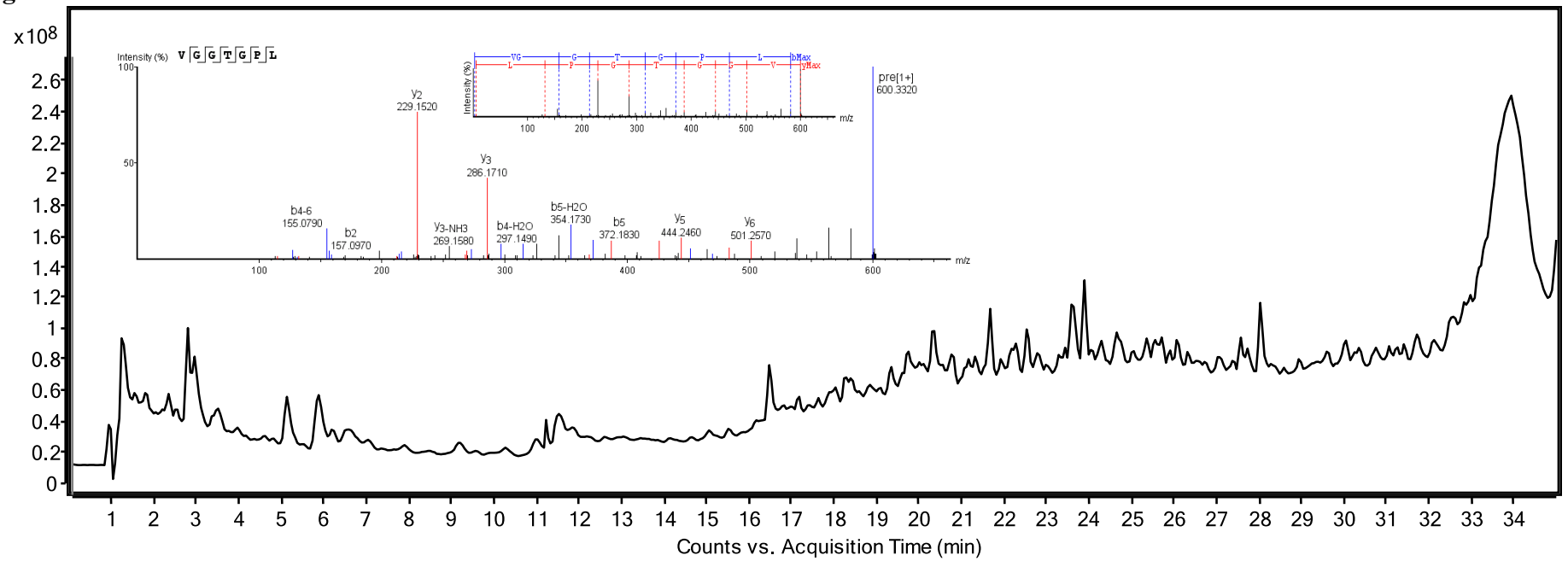

x10^8
Intensity (%)
V G G T G P L
y2
229.1520
y3
286.1710
pre[1+]
600.3320
b4-6
155.0790
b2
157.0970
y3-NH3
269.1580
b4-H2O
297.1490
b5-H2O
354.1730
b5
372.1830
y5
444.2460
y6
501.2570
m/z
Counts vs. Acquisition Time (min)

Figure 3.

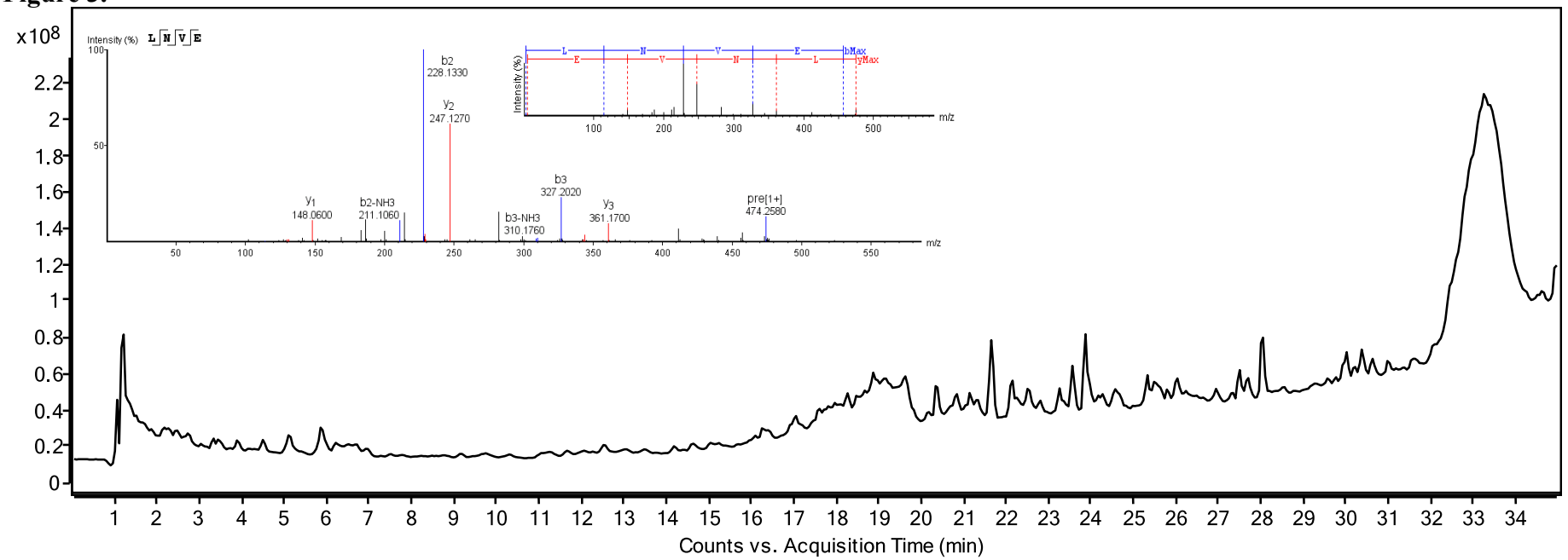

x10^8
Intensity (%)
L N V E
b2
228.1330
y2
247.1270
y1
148.0600
b2-NH3
211.1060
b3-NH3
310.1760
b3
327.2020
y3
361.1700
pre[1+]
474.2580
m/z
bMax
yMax
Counts vs. Acquisition Time (min)

Figure 4.

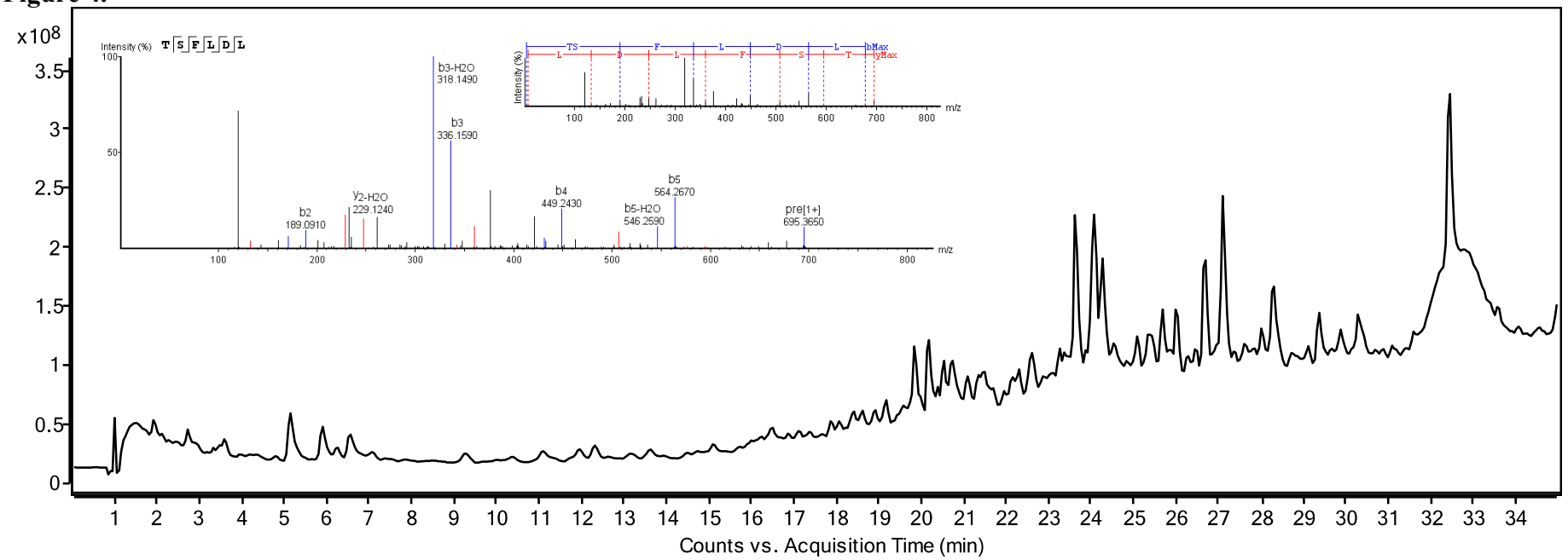

x10^8
3.5
3
2.5
2
1.5
1
0.5
0
Intensity (%)
T S F L D L
b3-H2O
318.1490
b3
336.1590
y2-H2O
229.1240
b2
189.0910
b4
449.2430
b5
564.2670
b5-H2O
546.2590
pre[1+]
695.3650
m/z
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
Counts vs. Acquisition Time (min)

Figure 5.

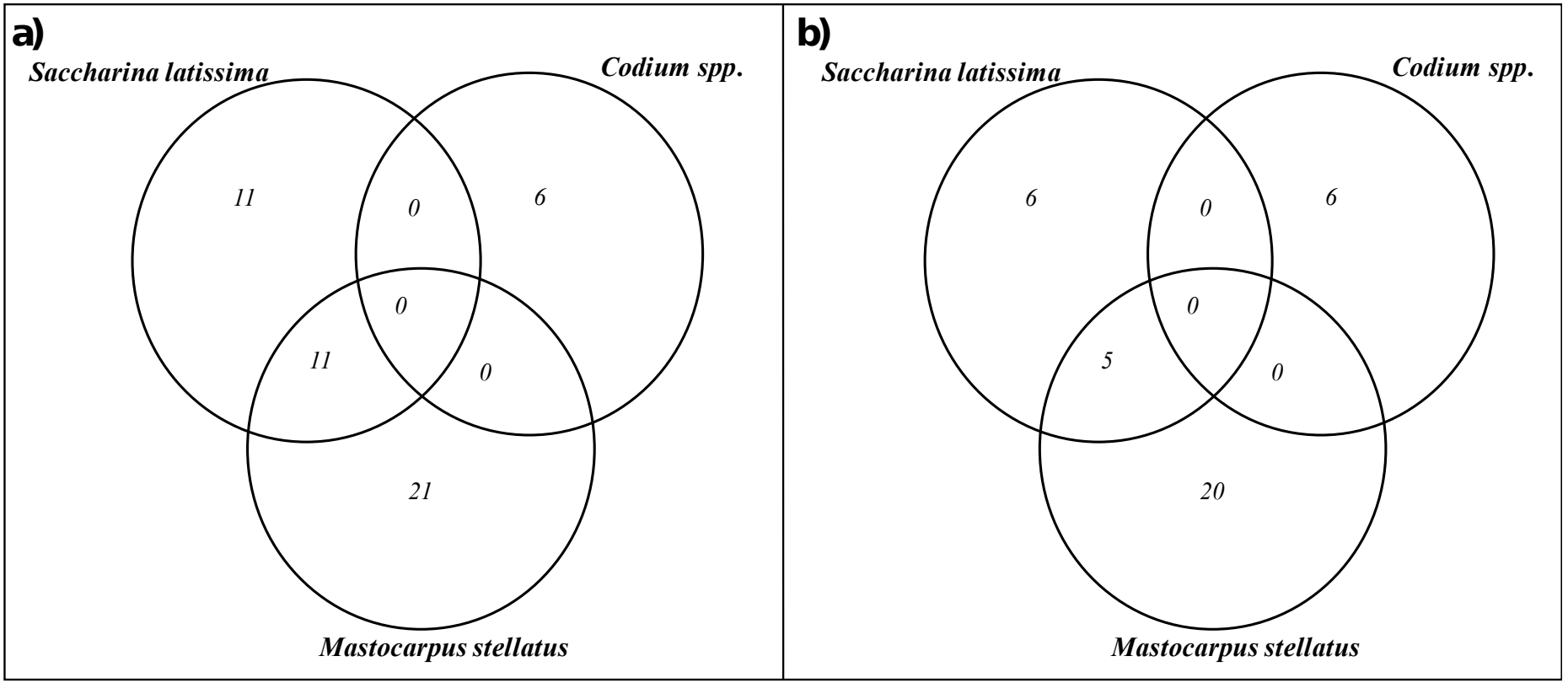

a)
Saccharina latissima
Codium spp.
11
0
6
0
11
0
21
Mastocarpus stellatus
b)
Saccharina latissima
Codium spp.
6
0
6
0
5
0
20
Mastocarpus stellatus

Table 1. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the alcalase hydrolysates of WPHCl, WPA, APHCl and APA protein extracts from *Mastocarpus stellatus* using RP-HPLC-ESI-QTOF-MS/MS and *de novo* sequencing tool.

				WPHCl		WPA		APHCl		APA		
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	LGDN	3.4	417.1859	-5 ± 2	91 ± 1	-7 ± 1	91 ± 1	-3 ± 2	91 ± 1	-	-	-
2	ATLN*	6.8	417.2224	-	-	-	-	-	-	-5 ± 3	89 ± 2	-
3	VVGGT	7.6	431.2380	-7 ± 2	89 ± 1	-	-	-	-	-	-	-
4	VVDT	9.1	432.2220	-	-	-	-	-5 ± 2	93 ± 1	-	-	-
5	SLGGAS	10.0	490.2387	-7 ± 1	90 ± 2	-7 ± 2	89 ± 2	-5 ± 2	90 ± 2	-5 ± 2	90 ± 2	-
6	AVVK	12.2	415.2794	-10 ± 1	93 ± 1	-	-	-9 ± 3	93 ± 1	-	-	-
7	VLNE	14.3	473.2485	-	-	-	-	-2 ± 2	87 ± 1	-	-	Antibacterial/membrane-active peptide/immunomodulating
8	LVQT	15.5	459.2693	-6 ± 1	88 ± 2	-	-	-	-	-	-	-
9	VLAE	16.5	430.2427	-	-	-	-	-3 ± 2	95 ± 1	-4 ± 2	92 ± 1	VIAE: Antibacterial
10	VTSLN	18.1	532.2857	-6 ± 1	93 ± 1	-	-	-	-	-	-	-
11	VVVE	18.1	444.2584	-7 ± 1	94 ± 2	-	-	-	-	-	-	-
12	VLVN	18.5	443.2744	-	-	-	-	-4 ± 2	88 ± 2	-	-	-
13	TLSAADAA	18.7	718.3497	-3 ± 1	92 ± 1	-	-	-	-	-	-	-
14	LNVE	18.9	473.2485	-	-	-4 ± 1	90 ± 2	-2 ± 2	90 ± 2	-3 ± 2	91 ± 2	-
15	VTSL	19.4	418.2427	-4 ± 1	91 ± 1	-4 ± 2	90 ± 2	-	-	-	-	Antibacterial
16	GTGPL	19.7	443.2380	-2 ± 2	88 ± 1	-	-	-3 ± 2	88 ± 2	-	-	-
17	ATYLGS*	19.8	610.2962	-	-	-4 ± 1	89 ± 2	-	-	-	-	-
18	APGAGVY*	20.3	633.3122	-1 ± 1	90 ± 1	-3 ± 1	89 ± 2	-2 ± 1	90 ± 2	-3 ± 2	89 ± 2	Antioxidant
19	LNVEAA	21.2	615.3228	-3 ± 1	89 ± 1	-	-	-	-	-	-	-
20	SVGAELE*	21.4	703.3388	-	-	-	-	-2 ± 2	95 ± 0	-	-	-
21	VGGTGPL	21.9	599.3279	-3 ± 1	94 ± 1	-	-	-	-	-	-	-
22	SVGAEL*	22.3	574.2962	-	-	-3 ± 1	91 ± 1	-	-	-	-	-
23	SLPLN	22.4	542.3064	-	-	-	-	-3 ± 2	91 ± 2	-	-	-
24	VLDTGLQ	22.8	744.4017	-	-	-	-	-	-	-3 ± 2	91 ± 2	-
25	VSLY*	23.2	480.2584	-3 ± 1	95 ± 1	-7 ± 2	93 ± 2	-2 ± 2	95 ± 1	-3 ± 3	93 ± 2	-

26	VAVL*	23.5	400.2686	-	-	-	-	-	-	-4 ± 3	91 ± 2	Antibacterial
27	VLDTGLQA	24.6	815.4388	-3 ± 1	87 ± 2	-	-	-	-	-3 ± 2	87 ± 2	-
28	FGPL	25.3	432.2372	-	-	-	-	-2 ± 1	88 ± 2	-4 ± 2	87 ± 1	FGPL/FGPI: stimulating
29	YLAL	25.9	478.2791	-6 ± 1	92 ± 1	-8 ± 2	89 ± 3	-	-	-	-	-
30	GLLNVE	26.0	643.3541	-	-	-2 ± 1	88 ± 2	-	-	-	-	-
31	MGDVLNM	27.5	778.3354	-	-	-3 ± 1	88 ± 2	-	-	-	-	--
32	LVLL	28.4	456.3311	-7 ± 2	90 ± 2	-	-	-	-	-	-	

***** Peptide that could belong to the alcalase enzyme protein sequence.

Table 2. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the alcalase hydrolysates of WPHCl, WPA, APHCl and APA protein extracts from *Saccharina latissima* using RP-HPLC-ESI-QTOF-MS/MS and *de novo* sequencing tool.

				WPHCl		WPA		APHCl		APA		
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	VAGAA*	4.5	387.2118	-	-	-	-	-	-	-2 ± 1	89 ± 1	-
2	SVGAE*	5.4	461.2122	-	-	-2 ± 1	95 ± 1	-	-	-2 ± 1	91 ± 2	-
3	QQQV	6.3	501.2547	-4 ± 2	87 ± 1	-	-	-	-	-	-	Stimulating different activities
4	ATLN*	6.8	417.2224	-5 ± 1	93 ± 1	-2 ± 1	93 ± 1	-	-	-3 ± 1	92 ± 1	-
5	SLGGAS	9.6	490.2387	-4 ± 1	93 ± 1	-1 ± 1	92 ± 2	-	-	-4 ± 1	91 ± 1	-
6	VVGQ	10.0	401.2274	-	-	-2 ± 1	87 ± 2	-	-	-	-	Antibacterial
7	DTGLQ	11.6	532.2493	-4 ± 1	88 ± 2	-1 ± 1	89 ± 2	-	-	-	-	-
8	YYGK*	13.1	529.2537	-	-	-	-	-	-	-3 ± 1	91 ± 3	
9	QQGL	15.7	444.2332	-4 ± 1	87 ± 1	-	-	-	-	-	-	-
10	LNVE	18.8	473.2485	-2 ± 1	94 ± 1	-2 ± 1	95 ± 1	-2 ± 2	92 ± 1	1 ± 1	92 ± 2	-
11	ASHPDLN*	19.7	752.3453	-	-	-1 ± 1	94 ± 2	-	-	-	-	-
12	ATYLGS*	19.8	610.2962	-1 ± 1	92 ± 2	-	-	-1 ± 1	91 ± 3	1 ± 0	91 ± 3	-
13	SHPDLN*	19.9	681.3082	-	-	1 ± 1	96 ± 1	-	-	-	-	-
14	APGAGVY*	20.3	633.3122	1 ± 1	93 ± 1	3 ± 0	94 ± 1	-	-	2 ± 1	92 ± 2	Antioxidant
15	LNVEAA	21.1	615.3228	-	-	-	-	-	-	1 ± 1	91 ± 2	-
16	SVGAELE*	21.4	703.3388	-	-	-1 ± 1	96 ± 1	-	-	-	-	-
17	VLDTGLQ	22.8	744.4017	-1 ± 0	95 ± 1	-	-	-	-	-	-	-
18	VSLY*	23.3	480.2584	-2 ± 1	97 ± 1	1 ± 1	97 ± 1	-	-	-1 ± 0	96 ± 1	-
19	VAVL*	23.5	400.2686	-3 ± 1	94 ± 1	-	-	-	-	-1 ± 1	92 ± 1	Antibacterial
20	LDLY	24.9	522.2689	-	-	-	-	-	-	-2 ± 2	93 ± 2	Antibacterial
21	MGDVLNM	27.4	778.3354	-1 ± 1	90 ± 2	-	-	-	-	-1 ± 1	89 ± 3	-
22	LGFL	28.3	448.2686	-	-	-	-	-	-	-6 ± 1	89 ± 2	Membrane -active peptide, antioxidant and ACE inhibitor

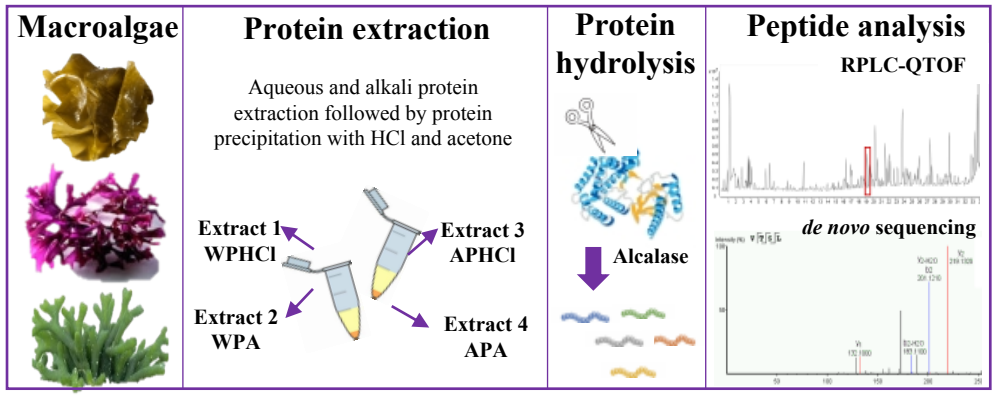
*Peptide that could belong to the alcalase enzyme protein sequence.

Table 3. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the alcalase hydrolysate of WPA protein extract from *Codium spp*. using RP-HPLC-ESI-QTOF-MS/MS and *de novo* sequencing tool.

		WPA				
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	NVVDGQPVLN	24.03	1053.5454	-12 ± 3	93 ± 1	-
2	APLDVGVD	24.25	784.3967	-11 ± 2	94 ± 1	-
3	GFGDGL	25.19	564.2543	-11 ± 3	90 ± 1	-
4	LPLVF	30.75	587.3682	-14 ± 1	92 ± 1	-
5	TSFLDL	30.87	694.3538	-12 ± 2	93 ± 1	-
6	FLPLVF	33.60	734.4366	-12 ± 2	94 ± 1	-

Highlights

- A LC-MS method was developed to separate and identify peptides in edible macroalgae
- Alcalase hydrolysates from three different macroalgae were analyzed.
- Thirty-seven different peptides were identified in the three macroalgae.
- Five of these peptides were common in brown and red macroalgae.
- Any of the peptides identified had previously been found in macroalgae.


Macroalgae
Protein extraction
Aqueous and alkali protein extraction followed by protein precipitation with HCl and acetone
Extract 1
WPHCl
Extract 3
APHCl
Extract 2
WPA
Extract 4
APA
Protein hydrolysis
Alcalase
Peptide analysis
RPLC-QTOF
de novo sequencing

SEPARATION AND IDENTIFICATION OF PEPTIDES IN HYDROLYSED PROTEIN EXTRACTS FROM EDIBLE MACROALGAE BY HPLC-ESI-QTOF/MS

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Tel: (34) 918894935

ABSTRACT

Macroalgae contain significant amounts of high-quality proteins which, because of their structural diversity, contain a range of yet undiscovered peptides within their primary structures. In this work, an analytical methodology was developed for the separation and identification of peptides present in protein hydrolysates from three different edible macroalgae used for human consumption (*Saccharina latissima* (brown macroalga), *Codium spp*. (green macroalga), and *Mastocarpus stellatus* (red macroalga)). The extraction of aqueous and alkaline soluble proteins was carried out followed by their precipitation with HCl or acetone. The protein extracts obtained were submitted to enzymatic digestion with alcalase and subsequently analyzed by reversed-phase high-performance liquid chromatography-quadrupole-time-of flight mass spectrometry (RP-HPLC-QTOF/MS) and *de novo* sequencing tool to separate and identify different short chain peptides. Thirty-seven peptides were identified in the hydrolysed protein extracts from the three macroalgae, five of them being common in brown and red macroalgae. After checking against BIOPEP database, several sequenced peptides were found within longer peptides with potential antibacterial activity. Any of the identified peptides had previously been identified in macroalgae.

Keywords: Peptides; reversed-phase; liquid chromatography-tandem mass spectrometry; macroalgae**.**

1. INTRODUCTION

Macroalgae are a diverse group of marine organisms which generate a wide group of functional biomolecules to survive under stress conditions [1]. They produce high-quality proteins whose concentrations can vary from 5 to 15% in the case of brown algae (Phaeophyta), from 9 to 26% for green algae (Chlorophyta) and from 10 to 47% for red algae (Rhodophyta) (percentages referred to dry weight) [2]. Peptides contained in proteins from marine sources, which can be released during enzymatic hydrolysis, food processing or ripening [3], have a high interest since they could present different type of bioactivity such as anti-cancerous, anti-proliferative, anti-coagulant, antibacterial, antifungal, and anti-tumor, among others [4-9]. Although peptides contained in protein hydrolysates from macroalgae could present some type of bioactivity, their separation and identification in these macroalgae protein hydrolysates have scarcely been investigated [10-15].

One of the most relevant challenges to obtain peptides from macroalgae is related to the extraction of proteins from the matrix since it is a topic which has not been studied deeper compared to the extraction of proteins from crops [16, 17] Protein extraction from macroalgae is a difficult task due to the cross-linking between polysaccharides and proteins within the matrix, as well as the inaccessibility of proteins within macromolecular cell wall assemblies [18]. The cross-linking between polysaccharides and proteins is especially important for brown macroalgae [19]. For instance, the extraction of proteins from the Laminaria alga *Saccharina japonica* has proved to be difficult due to the high levels of non-protein compounds (mainly viscous polysaccharides) whose presence interferes with protein extraction [20]. As a consequence, the main methods used for the extraction and precipitation of proteins are not completely useful in macroalgae.

High-performance liquid chromatography (HPLC) coupled to mass spectrometry (MS) is the analytical technique mainly employed to carry out the separation and identification of peptides [21]. Nowadays, most of the MS systems are able to detect with accuracy peptides with a length higher than 5 amino acids. However, the analysis of shorter peptides with 2 to 4 amino acids has scarcely been reported in the literature [21]. For instance, the low or high fragmentation of short peptides by tandem MS can make their detection difficult and challenging [21-23]. Thus, the development of analytical methods to carry out the separation and identification of short chain peptides presents a high interest when an in deep characterization of food is attempted.

The aim of this work was to separate and identify peptides contained in protein hydrolysates from three different edible macroalgae (*Saccharina latissima*, *Codium spp*. and *Mastocarpus stellatus*) used for human consumption. The extraction of aqueous and alkaline soluble proteins was carried out followed by protein precipitation using different approaches. Protein extracts obtained were subsequently submitted to enzymatic digestion and analyzed by reversed phase high-performance liquid chromatography coupled to a quadrupole-time-of flight mass spectrometer (RP-HPLC-QTOF/MS) and *de novo* sequencing tool.

2. MATERIALS AND METHODS

2.1 Chemicals and samples

All chemicals and reagents were of analytical grade. Sodium hydroxide, bovine serum albumin (BSA), and thermolysin were purchased from Sigma-Aldrich (Steinheim, Germany). Hydrochloric acid, acetone, methanol, ethanol and acetic acid were acquired in Scharlau (Barcelona, Spain). Sodium dodecyl sulfate (SDS) was purchased from Merck (Darmstadt, Germany). Alcalase 2.4 L FG was kindly donated by Novozymes

Spain S.A. (Madrid, Spain). Mini-protean precast gels, Laemmli buffer, Tris/glycine/SDS running buffer, precision plus protein standards (recombinant proteins expressed by Escherichia coli with molecular weights of 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa), silver stain kit, and Bradford reagent (Coomassie Blue G-250) were acquired from Bio-Rad (Hercules, CA, USA).

For the HPLC-MS/MS analysis, MS grade methanol and formic acid from Sigma-Aldrich were employed. The ultrapure water used was obtained from a Milli-Q (Millipore, Bedford, MA, USA) instrument.

Macroalgae samples (*Saccharina latissima*, *Codium spp*. and *Mastocarpus stellatus*) consisted in dried algae kindly donated by Porto-Muíños, S.L. (La Coruña, Spain). Once the macroalgae were collected, they were washed, dried at 30-35 ºC and grinded.

2.2 Total protein content

The protein content of the macroalgae was determined by the Kjeldahl method [24]. Nitrogen data were converted into protein values employing a conversion factor of 6.25 and were expressed as g per 100 g of dried macroalga. Analyses were performed in triplicate.

2.3 Extraction of proteins

The procedure used for the extraction of water and alkaline soluble proteins from milled dried macroalgae was based on the method described by Harnedy and FitzGerald (2015) [18] with some modifications (see Figure 1). In brief, 0.5 g of dried milled macroalgae powder was suspended in milli-Q water (1:20 (w/v)) and stirred gently for 3 h at 4 ºC. The proteins in the aqueous extract were removed by centrifugation at 4000 x g for 15 min at 4 ºC.

For alkaline soluble protein extraction, the pellet obtained after centrifugation was resuspended in 0.12 M NaOH at a weight volume ratio of 1:15 (w/v) and stirred gently at

room temperature for 1 h. Alkaline extraction was performed twice and both supernatants obtained by centrifugation at 4000 x g for 15 min at room temperature were combined.

Then, proteins from the aqueous and alkaline extracts were precipitated employing two different methods. First, the proteins were precipitated by adjusting the pH of each extract to around pH 3.5 using HCl. Aqueous protein extracts were kept for 30 min at 4 ºC while alkaline protein extracts were kept at room temperature to achieve the precipitation. The solutions were centrifuged at 4000 x g for 15 min at 4 ºC, and the pellets obtained were collected and dried by vacuum-drying. The proteins obtained were called aqueous or alkaline proteins precipitated with HCl (WPHCl and APHCl, respectively).

Secondly, the remaining proteins in both supernatants, aqueous and alkaline solutions after protein precipitation with HCl, were subjected to a second precipitation using cold acetone. The supernatants were diluted twice their volume in cold acetone and allowed then to stand for 1 h at -8 ºC. The solutions were centrifuged at 4000 x g for 15 min at 4 ºC, and the pellets obtained were over-night dried at room temperature. The proteins obtained were called aqueous or alkaline proteins precipitated with acetone (WPA and APA, respectively). The protein content for both aqueous and alkali extracts precipitated with HCl and acetone was estimated by Bradford assay [25]. WPHCl, APHCl, WPA and APA were ready to be subjected to protein digestion. Protein extraction for each algae was carried out in triplicate.

2.4 SDS-PAGE

Proteins were separated by SDS-PAGE using a Bio-Rad Mini-protean system (Hercules, CA, USA). Proteins solutions were mixed with Laemmli buffer containing 5 % (v/v) β-mercaptoethanol, followed by heating at 100 °C during 5 min and loaded into commercial Mini-PROTEAN TGX Precast Protein Gels from Bio-Rad (Hercules, CA, USA). Proteins were separated by applying 80 V for 5 min and 150 V until the separation was completed

using Tris/glycine/SDS as running buffer. Molecular markers of standard proteins with molecular weights from 10 to 250 kDa were also run. After separation, proteins were treated with a fixing solution of water/MeOH/acetic acid (50/40/10 % (v/v)) by shaking for 30 min and then with a second fixing solution water/EtOH/acetic acid (85/10/5 % (v/v)) twice for 15 min each. Gels were then treated with an oxidizer solution for 5 min and washed with water followed by the addition of the silver reagent and shaking during 20 min. Afterwards, the gel was washed for 1 min with water and developer solution was added. Reaction was stopped by adding 5 % acetic acid solution.

2.5 Protein digestion

Protein extracts obtained from macroalgae were hydrolyzed using the enzyme alcalase following a procedure previously optimized by our research team for the hydrolysis of proteins from plum by-products [26]. The protein extracts were dissolved in 5 mM borate buffer (pH = 8.5) at a final concentration of 5 mg/mL with the help of an ultrasonic probe for 5-10 min and with 30 % of wave amplitude. Then, the enzyme was added at an enzyme/substrate ratio of 0.15 AU/g protein and the solution was incubated in a Thermomixer Compact (Eppendorf AG, Hamburg, Germany) at 50 ºC with agitation (700 rpm) for 4h. The digestion was stopped (100 ºC for 10 min) using Thermomixer Compact and the solution was centrifuged for 10 min at 6000 g. Finally, the supernatant was collected for its analysis by HPLC-ESI-MS/MS.

2.6 Separation and identification of peptides by RP-HPLC-ESI-QTOF/MS

Peptide analysis was performed using an HPLC system 1100 from Agilent (Agilent Technologies, Santa Clara, CA, USA) coupled to a quadrupole-time-of flight mass spectrometer (QTOF/MS) Agilent 6530 equipped with an orthogonal electrospray ionization (ESI) source (Agilent Jet Stream, AJS). The HPLC instrument was equipped with a quaternary solvent pump, an auto-sampler, and a column heater compartment.

Agilent Mass Hunter Workstation software B.07.00 from Agilent was employed for HPLC and MS control, data acquisition, and data analysis.

The separation was carried out using a porous-shell fused-core Ascentis Express C18 analytical column (150 mm x 2.1 mm, particle size 2.7 μm) with an Ascentis Express C18 guard column (0.5 cm × 2.1 mm, 2.7 μm particle size), both from Supelco (Bellefonte, Pa, USA). The column temperature was 50 ºC and the flow rate 300 μL/min. Five μL of extract were injected. The mobile phases consisted of (A) water with 0.5 % formic acid and (B) methanol with 0.5 % formic acid in a gradient elution analysis programmed as follows: 0 min, 1 % (B); 0-5 min, 1 % (B); 5-10 min, 1-5 % (B); 10-30 min, 5-60 % (B); 30-35 min, 60 % (B), with 15 min of post-time.

The mass spectrometer was operated in positive ion mode and the mass range was from 100 to 1700 m/z. MS parameters were the following: capillary voltage, 3500 V; nebulizer pressure, 50 psig; drying gas flow rate, 12 L/min; gas temperature, 350 ºC. The fragmentor voltage (cone voltage after capillary) was set at 80 V. The skimmer and octapole voltage were 60 V and 750 V, respectively. Source sheath gas temperature and flow were 400 ºC and 12 L/min, respectively. MS/MS was performed employing the auto mode and the following optimized conditions; 1 precursor per cycle, dynamic exclusion after two spectra (released after 1 min), and collision energy of 5 V for every 100 Da. Internal mass calibration of the instrument was carried out using an AJS ESI source with an automated calibrant delivery system. The reference compound solution for internal mass calibration containing purine and HP-0921 (hexakis(1H,1H,3H-tetrafluoropropoxy)phosphazine) in acetonitrile-water (90:10, v/v) (4 μM and 2.5 μM, respectively, 15 μL/min) from Agilent was used, *m/z* 121.0509 and *m/z* 922.0098, respectively. The analyses were conducted in triplicate.

Tandem MS/MS spectra were obtained for the molecular ion with the highest abundance. Every sample was injected in triplicate into the MS system. In order to assure that identified peptides came from macroalgae protein, MS/MS spectra were analyzed using PEAKS Studio Version 7 (Bioinformatics Solutions Inc., Waterloo, Canada). Data analysis was performed by *de novo* sequencing tool. Only those peptides identified with an ALC (expected percentage of correct amino acids in the peptide sequence) above 85% and with a good precursor fragmentation pattern were considered. Moreover, only those peptides appearing in at least 7 injections from 9 injections (three injections of each triplicate) were taken into account. Only isoforms with leucine (L) are presented in our results, although peptide sequences containing isoleucine (I) amino acid instead of L are also possible since it is not possible to differentiate I from L by the MS used.

3. RESULTS AND DISCUSSION

3.1. Development of an analytical methodology for the separation and identification of peptides by RP-HPLC-ESI-QTOF/MS

To achieve the separation and identification of peptides in hydrolysates from macroalgae protein extracts, an adequate analytical methodology, based on the use of HPLC-MS/MS, was developed. Taking into account that the protein contents for the three studied macroalgae (determined as described in section 2.1) were 6.3 ± 0.1 % for *Saccharina latissima*, 12.4 ± 0.8 % for *Codium spp*., and 16.9 ± 0.5 % for *Mastocarpus stellatus* (all percentages referred to sample dry weight), the macroalga *M. stellatus* was selected to perform the optimization of the chromatographic and MS parameters due to its higher protein content. Then, a protein aqueous extract was obtained and precipitated with HCl (WPHCl) following the protocol previously described (see section 2.3). The protein extract obtained was hydrolyzed and analyzed by HPLC-MS/MS using a C18 column. To

optimize the separation conditions, the effect of different parameters such as gradient program (gradient time, gradient shape, and initial composition of the mobile phase), column temperature (25-50 °C), flow rate (0.2-0.4 mL/min), and injection volume (2-5 µL), was investigated. The best resolution and shortest analysis time were achieved using a gradient elution based on water with 0.5 % formic acid (solvent A) and methanol with 0.5 % formic acid (solvent B) programmed as follows: 0 min, 1% B; 0-5 min, 1% B; 5-10 min, 1-5% B; 10-30 min, 5-60 % B; 30-35 min, 60% B, with 15 min of post-time at final composition. The other selected experimental conditions were a flow rate of 0.3 mL/min, a column temperature of 50 °C, and an injection volume of 5 µL. MS/MS parameters for peptide identification were selected taking into account those previously employed to identify peptides from different sources such as food and food by-products [26-28]. MS/MS data obtained using the developed method were analyzed using the *de novo* sequencing tool from the PEAK Software.

Figure 2 shows the Total Ion Chromatogram (TIC) corresponding to the analysis of protein hydrolysates from *M. stellatus* and the mass spectrum showing the fragmentation pattern of peak at 21.9 min and 600.3320 m/z (VGGTGPL peptide). As it can be observed, a good chromatographic profile could be obtained in an analysis time of 35 min.

3.2. Protein extraction and digestion

Protein extracts were obtained following the protocol described by Harnedy and FitzGerald (2015) [18] with some modifications. The method involved two subsequent aqueous and alkaline extractions under the conditions described in section 2.3. Protein profiles obtained by SDS-PAGE were obtained and compared for the three algae showing electrophoretic profiles with bands at molecular mass values lower than 25 kDa for *M. stellatus* and intense bands corresponding to molecular mass between 75 and 250 kDa

(see **Figure S1 from supporting information**) for the other two macroalgae. The total protein content (expressed in %) obtained from four extracts (WPHCl, APHCl, WPA and APA) from each macroalgae, estimated by Bradford assay, was 1.8 ± 0.5 % for *Saccharina latissima*, 2.7 ± 0.5 % for *Codium spp*., and 4.7 ± 0.7 % for *Mastocarpus stellatus.* showing higher extraction yields for the red and green macroalgae since these macroalgae presented higher crude protein content before extraction. Protein extracts obtained were precipitated with HCl and digested with the enzyme alcalase. Under these conditions, 12 and 17 peptides were found in aqueous extracts (WPHCl) and 2 and 14 peptides in alkaline extracts (APHCl) from *S. latissimi* and *M. stellatus*, respectively. However, peptides were not obtained from both extracts from *Codium spp*. Moreover, a gel formation was observed in *S. latissima* after the precipitation of proteins with HCl. This fact could be explained by the high levels of non-digestible viscous polysaccharides that make especially problematic the extraction of proteins from brown macroalgae [20, 29].

Thus, based on the experience of our research group on the analysis of peptides from different sources, protein precipitation was carried out using cold acetone instead of HCl (see section 2.3) [30]. Under these conditions, 11, 6, and 11 peptides were obtained in aqueous extracts from *S. latissimi*, *Codium spp*., and *M. stellatus,* respectively, and 14 and 10 peptides in alkaline extracts of *S. latissimi* and *M. stellatus* (peptides were not found in the alkaline extract for *Codium spp)*. Bearing in mind the results obtained with HCl and acetone, a combination of both approaches was evaluated. Thus, aqueous and alkaline extracts were firstly precipitated with HCl by adjusting the pH to around 3.5 (extracts WPHCl and APHCl, respectively). Then, the supernatants obtained were submitted to a second precipitation by adding cold acetone (extracts WPA and APA, respectively) (see Figure 1). Following this procedure, 12, 2, 11 and 14 peptides were

obtained in WPHCl, APHCL, WPA and APA extracts, respectively, from *S. latissima*; 6 peptides were detected in WPA extracts while any peptide was not found in WPHCl, APHCL and APA extracts from *Codium spp*., and 17, 14, 11 and 10 peptides were got in WPHCl, APHCL, WPA and APA extracts from the macroalga *M. stellatus,* respectively (see Tables 1, 2 and 3). Since a higher number of peptides could be obtained following this approach, it was selected to carry out the isolation of proteins from the different macroalgae.

Although two different enzymes, alcalase and thermolysin, were tested for protein digestion under the experimental conditions previously employed by our research team [26, 31], alcalase was chosen to achieve the hydrolysis since most of the peptides obtained using thermolysin belong to the protein sequence of this enzyme.

3.3. Peptide identification in protein hydrolysates

In order to carry out the tentative identification of peptides in the hydrolyzed protein extracts from *S. latissima*, *Codium spp*., and *M. stellatus*, they were analyzed by the developed HPLC-MS/MS method. Then, MS/MS data were treated by the PEAKS software to obtain *de novo* sequence.

Figures 2, 3 and 4 show the TIC chromatograms corresponding to the protein hydrolysates from WPHCl extract in *M. stellatus,* APA extract in *S. latissima* and APA extract in *Codium spp.,* respectively. These selected hydrolysates extracts presented the highest number of peptides for each macroalga. Moreover, these figures also display as an example, the mass spectrum with the fragmentation pattern of VGGTGPL, LNVE and TSFLDL peptides, respectively.

Tables 1, 2 and 3 show the different peptides identified in *M. stellatus, S. latissima* and *Codium spp*., respectively, along with their experimental molecular masses, ALC, and

accuracy. Forty-nine different peptides with a number of amino acids ranging from 4 to 10 were identified. As it can be seen in Figure 5, the Venn diagram showed eleven common peptides in *S. latissima* and *M. stellatus* (ATLN, SLGGAS, LNVE, ATYLGS, APGAGVY, LNVEAA, SVGAELE, VLDTGLQ, VSLY, VAVL, and MGDVLNM) and non-common peptides with *Codium spp*. (see Figure 5a and Tables 1, 2 and 3). Twelve peptides found in *S. latissima* and *M. stellatus* (VAGAA, SVGAE, ATLN, YYGK, ASHPDLN, ATYLGS, SHPDLN, APGAGVY, SVGAELE, VSLY, VAVL, SVGAEL) could belong to the alcalase enzyme sequence. Thus, thirty-seven different peptides were found in the three macroalgae being five of them common in *S. latissima* and *M. stellatus* (SLGGAS, LNVE, LNVEAA, VLDTGLQ, and MGDVLNM) (see Figure 5b).

The data obtained demonstrated that the highest number of peptides were found in the red macroalga *M. stellatus* (25 peptides), followed by the brown (*S. latissima*) (11 peptides) and green (*Codium spp*.) (6 peptides) macroalgae. Moreover, to the best of our knowledge, this is the first time that these peptides have been found in these macroalgae. The amino acid composition of the identified peptides in these macroalgae had high percentage of hydrophobic amino acids (leucine (L)/isoleucine (I), proline (P) and valine (V)) within their sequences.

In order to know the potential bioactivity of the identified peptides found in these macroalgae, they were verified against BIOPEP database [32]. Several sequences of peptides were found within longer peptides with potential bioactivities (see Tables 1, 2 and 3). For instance, the peptides VLNE, VIAE, VTSL, VVGQ and LDLY were previously found within a longer sequence of antibacterial peptides. However, most of the peptides found in these macroalgae have not previously been reported.

4. CONCLUSIONS

An analytical methodology was developed for the first time enabling the separation and identification of short chain peptides from three edible macroalgae, *M. stellatus, S. latissima* and *Codium spp*. The extraction of aqueous and alkaline soluble proteins was achieved followed by their precipitation and enzymatic hydrolysis with alcalase enzyme. Peptide hydrolysates were analyzed by HPLC-MS/MS and *de novo* sequenced using PEAKS software. Thirty-seven peptides were identified in the three macroalgae, being five of them common in *M. stellatus* and *S. latissima*. The peptides identified in these samples were not previously found in macroalgae. After checking against BIOPEP database, several sequenced peptides were found within longer peptides with potential bioactivities mainly with antibacterial properties.

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Declaration of author contributions

All authors were involved in the conception and design of the study. R.P.M performed most experimental work and data acquisition. M.L.M. was responsible for getting financial support. All the authors contributed to data interpretation and the writing of the manuscript, performed its critical revision and approved the final manuscript.

Conflicts of interest

The authors have no conflict of interest to disclose.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable

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FIGURE CAPTIONS

Fig. 1 Protein extraction procedure employed in this work.

Fig. 2 Total ion chromatogram from WPHCl extracts hydrolysed with alcalase from *Mastocarpus stellatus* by RP-HPCL-ESI-QTOF-MS/MS and an example of MS/MS spectrum of the peptide VGGTGPL observed at 21.9 min (molecular mass (Da): 599.3279).

Fig. 3 Total ion chromatogram from APA extracts hydrolysed with alcalase from *Saccharina latissima* by RP-HPLC-ESI-QTOF-MS/MS and an example of MS/MS spectrum of the peptide LNVE observed at 18.8 min (molecular mass (Da): 473.2485).

Fig. 4 Total ion chromatogram from WPA extracts hydrolysed with alcalase from *Codium spp*. by RP-HPLC-ESI-QTOF-MS/MS and an example of MS/MS spectrum of the peptide TSFLDL observed at 30.9 min (molecular mass (Da): 694.3538).

Fig. 5 Venn diagram of peptides identified in *Mastocarpus stellatus, Saccharina latissima* and *Codium spp* taking into account the peptides in common with the alcalase enzyme protein sequence (a) and without taking into consideration the peptides in common with the alcalase enzyme protein sequence (b).

Figure 1.

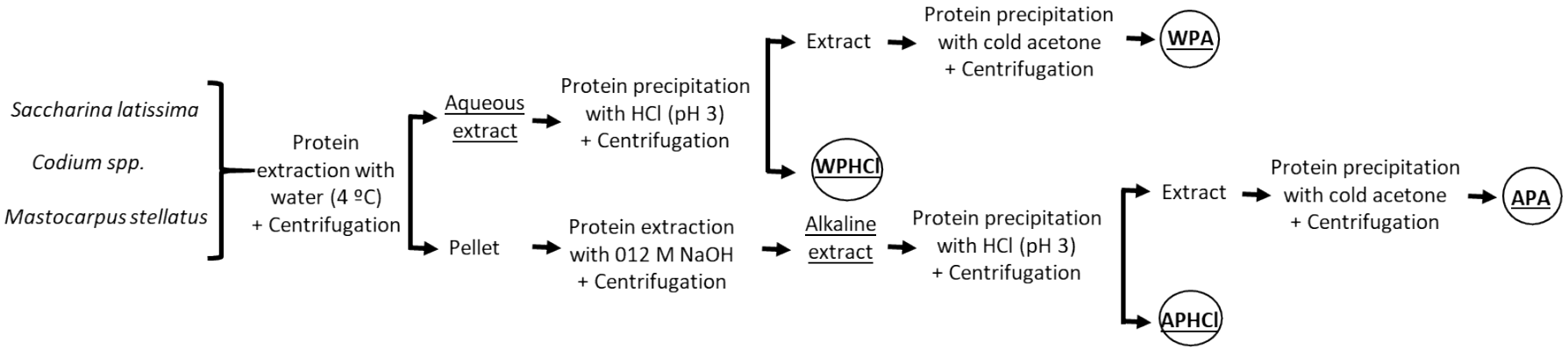
Saccharina latissima
Codium spp.
Mastocarpus stellatus
Protein extraction with water (4 ºC) + Centrifugation
Aqueous extract
Protein precipitation with HCl (pH 3) + Centrifugation
Extract
Protein precipitation with cold acetone + Centrifugation
WPA
WPHCl
Pellet
Protein extraction with 012 M NaOH + Centrifugation
Alkaline extract
Protein precipitation with HCl (pH 3) + Centrifugation
Extract
Protein precipitation with cold acetone + Centrifugation
APA
APHCl

Figure 2.

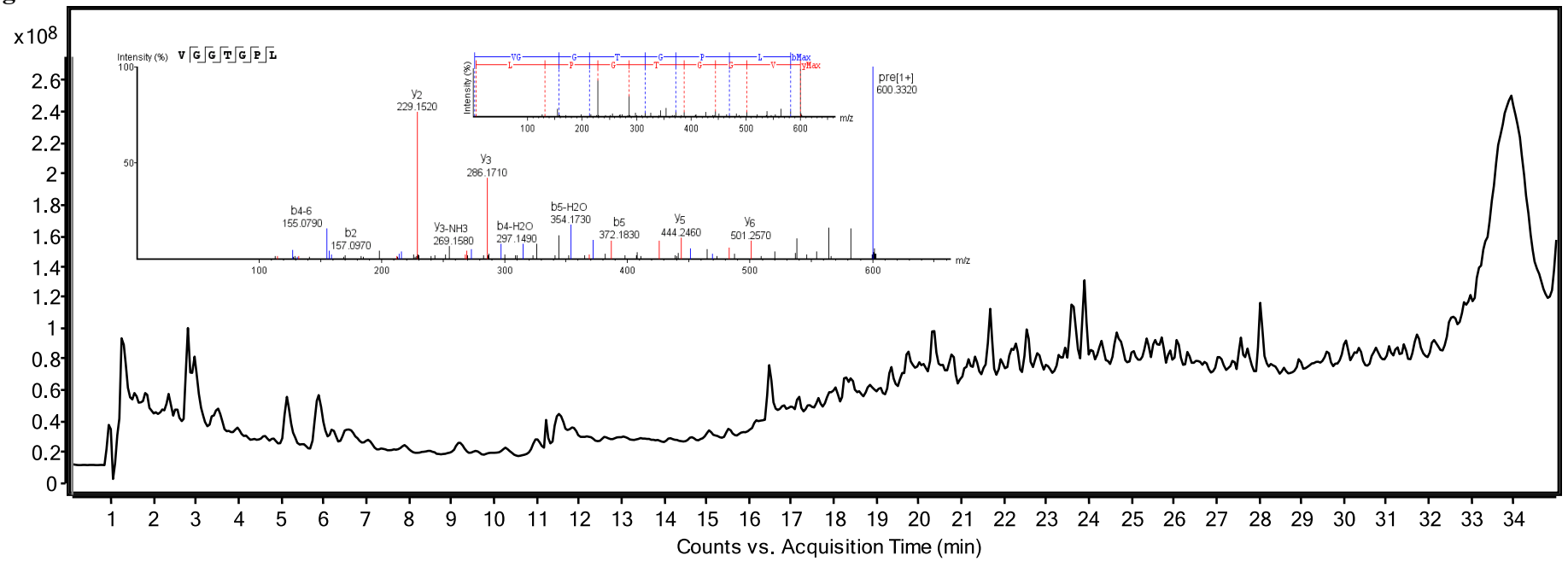
x10^8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Intensity (%)
V G G T G P L
y2
229.1520
y3
286.1710
b4-6
155.0790
b2
157.0970
y3-NH3
269.1580
b4-H2O
297.1490
b5-H2O
354.1730
b5
372.1830
y5
444.2460
y6
501.2570
pre[1+]
600.3320
m/z
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
Counts vs. Acquisition Time (min)

Figure 3.

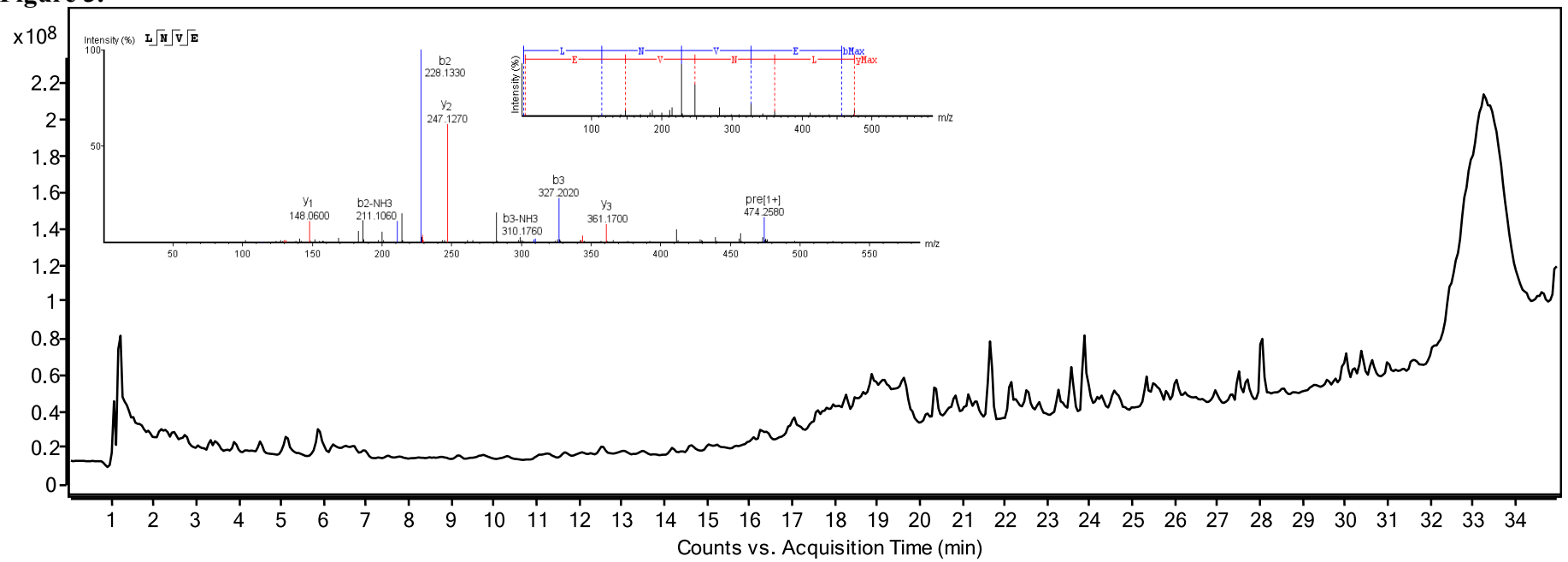
x10^8
Intensity (%)
L N V E
b2
228.1330
y2
247.1270
y1
148.0600
b2-NH3
211.1060
b3-NH3
310.1760
b3
327.2020
y3
361.1700
pre[1+]
474.2580
m/z
bMax
yMax
Counts vs. Acquisition Time (min)

Figure 4.

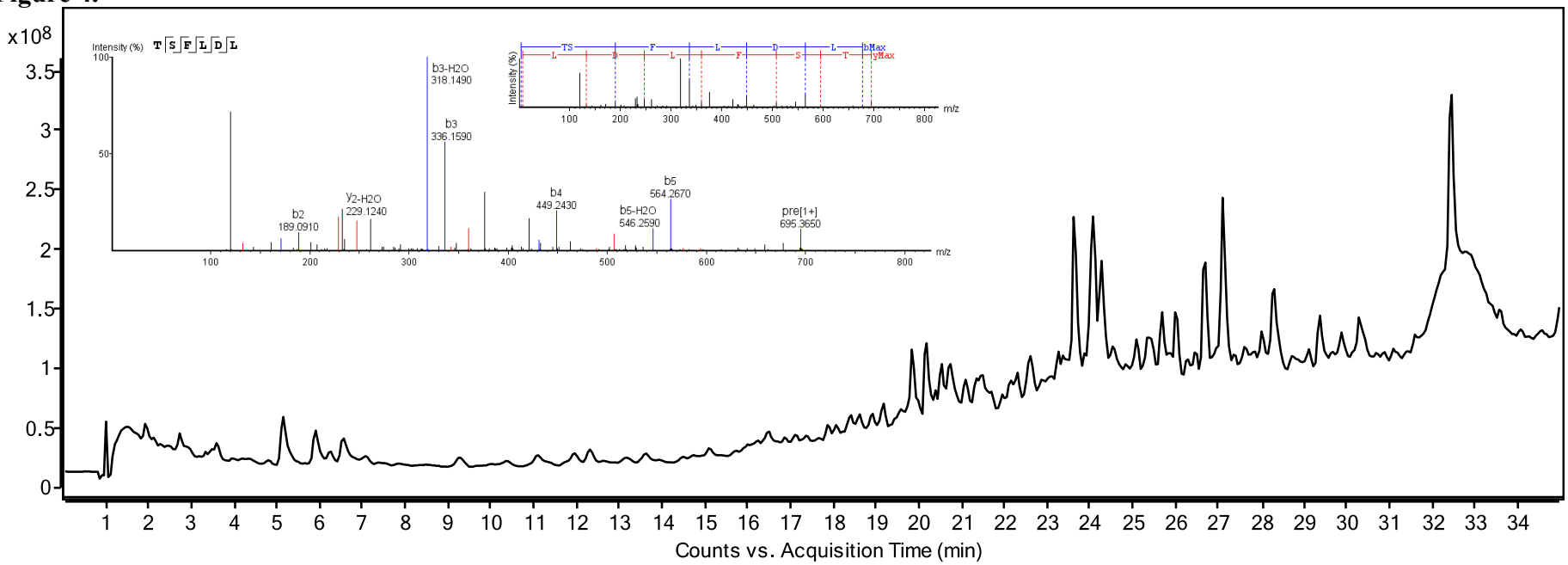
x10^8
3.5
3
2.5
2
1.5
1
0.5
0
Intensity (%)
T S F L D L
b3-H2O
318.1490
b3
336.1590
y2-H2O
229.1240
b2
189.0910
b4
449.2430
b5
564.2670
b5-H2O
546.2590
pre[1+]
695.3650
m/z
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34
Counts vs. Acquisition Time (min)

Figure 5.

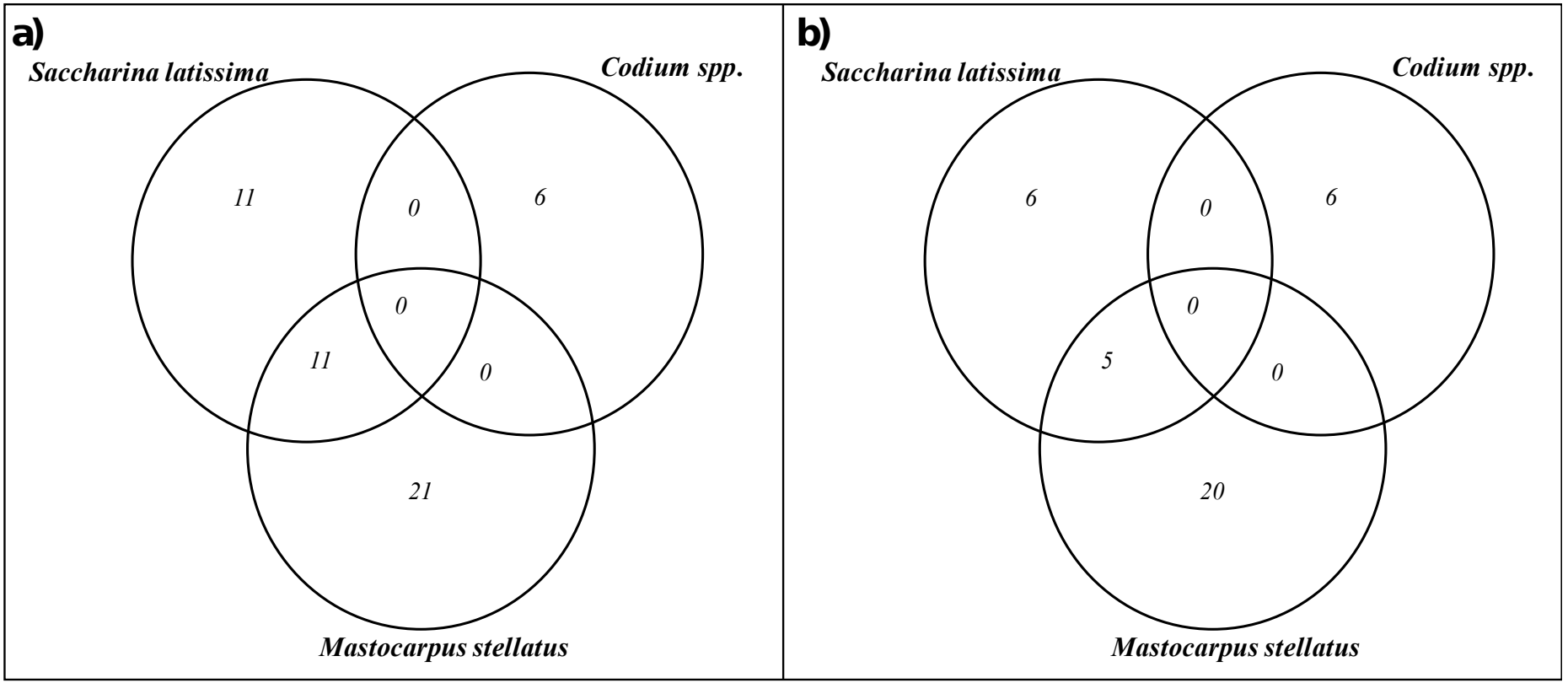
a)
Saccharina latissima
Codium spp.
11
0
6
0
11
0
21
Mastocarpus stellatus
b)
Saccharina latissima
Codium spp.
6
0
6
0
5
0
20
Mastocarpus stellatus

Table 1. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the alcalase hydrolysates of WPHCl, WPA, APHCl and APA protein extracts from *Mastocarpus stellatus* using RP-HPLC-ESI-QTOF-MS/MS and *de novo* sequencing tool.

				WPHCl		WPA		APHCl		APA		
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	LGDN	3.4	417.1859	-5 ± 2	91 ± 1	-7 ± 1	91 ± 1	-3 ± 2	91 ± 1	-	-	-
2	ATLN*	6.8	417.2224	-	-	-	-	-	-	-5 ± 3	89 ± 2	-
3	VVGGT	7.6	431.2380	-7 ± 2	89 ± 1	-	-	-	-	-	-	-
4	VVDT	9.1	432.2220	-	-	-	-	-5 ± 2	93 ± 1	-	-	-
5	SLGGAS	10.0	490.2387	-7 ± 1	90 ± 2	-7 ± 2	89 ± 2	-5 ± 2	90 ± 2	-5 ± 2	90 ± 2	-
6	AVVK	12.2	415.2794	-10 ± 1	93 ± 1	-	-	-9 ± 3	93 ± 1	-	-	-
7	VLNE	14.3	473.2485	-	-	-	-	-2 ± 2	87 ± 1	-	-	Antibacterial/membrane-active peptide/immunomodulating
8	LVQT	15.5	459.2693	-6 ± 1	88 ± 2	-	-	-	-	-	-	-
9	VLAE	16.5	430.2427	-	-	-	-	-3 ± 2	95 ± 1	-4 ± 2	92 ± 1	VIAE: Antibacterial
10	VTSLN	18.1	532.2857	-6 ± 1	93 ± 1	-	-	-	-	-	-	-
11	VVVE	18.1	444.2584	-7 ± 1	94 ± 2	-	-	-	-	-	-	-
12	VLVN	18.5	443.2744	-	-	-	-	-4 ± 2	88 ± 2	-	-	-
13	TLSAADAA	18.7	718.3497	-3 ± 1	92 ± 1	-	-	-	-	-	-	-
14	LNVE	18.9	473.2485	-	-	-4 ± 1	90 ± 2	-2 ± 2	90 ± 2	-3 ± 2	91 ± 2	-
15	VTSL	19.4	418.2427	-4 ± 1	91 ± 1	-4 ± 2	90 ± 2	-	-	-	-	Antibacterial
16	GTGPL	19.7	443.2380	-2 ± 2	88 ± 1	-	-	-3 ± 2	88 ± 2	-	-	-
17	ATYLGS*	19.8	610.2962	-	-	-4 ± 1	89 ± 2	-	-	-	-	-
18	APGAGVY*	20.3	633.3122	-1 ± 1	90 ± 1	-3 ± 1	89 ± 2	-2 ± 1	90 ± 2	-3 ± 2	89 ± 2	Antioxidant
19	LNVEAA	21.2	615.3228	-3 ± 1	89 ± 1	-	-	-	-	-	-	-
20	SVGAELE*	21.4	703.3388	-	-	-	-	-2 ± 2	95 ± 0	-	-	-
21	VGGTGPL	21.9	599.3279	-3 ± 1	94 ± 1	-	-	-	-	-	-	-
22	SVGAEL*	22.3	574.2962	-	-	-3 ± 1	91 ± 1	-	-	-	-	-
23	SLPLN	22.4	542.3064	-	-	-	-	-3 ± 2	91 ± 2	-	-	-
24	VLDTGLQ	22.8	744.4017	-	-	-	-	-	-	-3 ± 2	91 ± 2	-
25	VSLY*	23.2	480.2584	-3 ± 1	95 ± 1	-7 ± 2	93 ± 2	-2 ± 2	95 ± 1	-3 ± 3	93 ± 2	-

26	VAVL*	23.5	400.2686	-	-	-	-	-	-	-4 ± 3	91 ± 2	Antibacterial
27	VLDTGLQA	24.6	815.4388	-3 ± 1	87 ± 2	-	-	-	-	-3 ± 2	87 ± 2	-
28	FGPL	25.3	432.2372	-	-	-	-	-2 ± 1	88 ± 2	-4 ± 2	87 ± 1	FGPL/FGPI: stimulating
29	YLAL	25.9	478.2791	-6 ± 1	92 ± 1	-8 ± 2	89 ± 3	-	-	-	-	-
30	GLLNVE	26.0	643.3541	-	-	-2 ± 1	88 ± 2	-	-	-	-	-
31	MGDVLNM	27.5	778.3354	-	-	-3 ± 1	88 ± 2	-	-	-	-	--
32	LVLL	28.4	456.3311	-7 ± 2	90 ± 2	-	-	-	-	-	-	

* Peptide that could belong to the alcalase enzyme protein sequence.

Table 2. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the alcalase hydrolysates of WPHCl, WPA, APHCl and APA protein extracts from *Saccharina latissima* using RP-HPLC-ESI-QTOF-MS/MS and *de novo* sequencing tool.

				WPHCl		WPA		APHCl		APA		
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	VAGAA*	4.5	387.2118	-	-	-	-	-	-	-2 ± 1	89 ± 1	-
2	SVGAE*	5.4	461.2122	-	-	-2 ± 1	95 ± 1	-	-	-2 ± 1	91 ± 2	-
3	QQQV	6.3	501.2547	-4 ± 2	87 ± 1	-	-	-	-	-	-	Stimulating different activities
4	ATLN*	6.8	417.2224	-5 ± 1	93 ± 1	-2 ± 1	93 ± 1	-	-	-3 ± 1	92 ± 1	-
5	SLGGAS	9.6	490.2387	-4 ± 1	93 ± 1	-1 ± 1	92 ± 2	-	-	-4 ± 1	91 ± 1	-
6	VVGQ	10.0	401.2274	-	-	-2 ± 1	87 ± 2	-	-	-	-	Antibacterial
7	DTGLQ	11.6	532.2493	-4 ± 1	88 ± 2	-1 ± 1	89 ± 2	-	-	-	-	-
8	YYGK*	13.1	529.2537	-	-	-	-	-	-	-3 ± 1	91 ± 3	
9	QQGL	15.7	444.2332	-4 ± 1	87 ± 1	-	-	-	-	-	-	-
10	LNVE	18.8	473.2485	-2 ± 1	94 ± 1	-2 ± 1	95 ± 1	-2 ± 2	92 ± 1	1 ± 1	92 ± 2	-
11	ASHPDLN*	19.7	752.3453	-	-	-1 ± 1	94 ± 2	-	-	-	-	-
12	ATYLGS*	19.8	610.2962	-1 ± 1	92 ± 2	-	-	-1 ± 1	91 ± 3	1 ± 0	91 ± 3	-
13	SHPDLN*	19.9	681.3082	-	-	1 ± 1	96 ± 1	-	-	-	-	-
14	APGAGVY*	20.3	633.3122	1 ± 1	93 ± 1	3 ± 0	94 ± 1	-	-	2 ± 1	92 ± 2	Antioxidant
15	LNVEAA	21.1	615.3228	-	-	-	-	-	-	1 ± 1	91 ± 2	-
16	SVGAELE*	21.4	703.3388	-	-	-1 ± 1	96 ± 1	-	-	-	-	-
17	VLDTGLQ	22.8	744.4017	-1 ± 0	95 ± 1	-	-	-	-	-	-	-
18	VSLY*	23.3	480.2584	-2 ± 1	97 ± 1	1 ± 1	97 ± 1	-	-	-1 ± 0	96 ± 1	-
19	VAVL*	23.5	400.2686	-3 ± 1	94 ± 1	-	-	-	-	-1 ± 1	92 ± 1	Antibacterial
20	LDLY	24.9	522.2689	-	-	-	-	-	-	-2 ± 2	93 ± 2	Antibacterial
21	MGDVLNM	27.4	778.3354	-1 ± 1	90 ± 2	-	-	-	-	-1 ± 1	89 ± 3	-
22	LGFL	28.3	448.2686	-	-	-	-	-	-	-6 ± 1	89 ± 2	Membrane -active peptide, antioxidant and ACE inhibitor

*Peptide that could belong to the alcalase enzyme protein sequence.

Table 3. Peptide sequence, retention time (RT), molecular mass, mass accuracy, average local confidence (ALC) and activity described in BIOPEP (2017) database of the peptides identified in the alcalase hydrolysate of WPA protein extract from *Codium spp*. using RP-HPLC-ESI-QTOF-MS/MS and *de novo* sequencing tool.

		WPA				
ID	**Peptide sequence**	**RT (min)**	**Molecular mass (Da)**	**Mass accuracy (ppm)**	**ALC (%)**	**Activity (BIOPEP database)**
1	NVVDGQPVLN	24.03	1053.5454	-12 ± 3	93 ± 1	-
2	APLDVGVD	24.25	784.3967	-11 ± 2	94 ± 1	-
3	GFGDGL	25.19	564.2543	-11 ± 3	90 ± 1	-
4	LPLVF	30.75	587.3682	-14 ± 1	92 ± 1	-
5	TSFLDL	30.87	694.3538	-12 ± 2	93 ± 1	-
6	FLPLVF	33.60	734.4366	-12 ± 2	94 ± 1	-

AUTHOR DECLARATION TEMPLATE

We wish to draw the attention of the Editor to the following facts which may be considered as potential conflicts of interest and to significant financial contributions to this work. [OR] We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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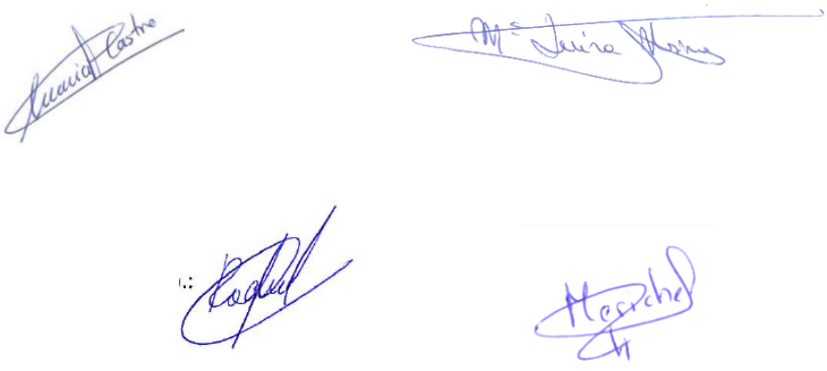

Supporting information

SEPARATION AND IDENTIFICATION OF PEPTIDES IN HYDROLYSED PROTEIN EXTRACTS FROM EDIBLE MACROALGAE BY HPLC-ESI-QTOF/MS

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Figure S1. SDS-PAGE gels corresponding to the protein extracts obtained for WPHCL, WPA, APHCL and APA extracts for each algae.