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Effects of high hydrostatic pressure and polysaccharidases on the extraction of antioxidant compounds from red macroalgae, *Palmaria palmata* and *Solieria chordalis*

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HHP
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ACE
Angiotensin-converting-enzyme
EAE
Enzyme-assisted extraction
MAE
Microwave-assisted extraction
UAE
Ultrasound-assisted extraction
PLE
Pressurized liquid extraction
AAPH
2,2′-azobis (2-amidinopropane) dihydrochloride
HCl
Hydrochloric acid
NaOH
Sodium hydroxide
C
Cellulase
H
Hemicellulase
ORAC
Oxygen radical absorbance capacity
AWA
Acetone/water/acetic acid
TE
Trolox equivalent

ABSTRACT

A non-thermal high hydrostatic pressure (HHP) technology in combination with polysaccharidases is proposed as a novel approach to improve the phytochemical extraction from red macroalgae. Two macroalgae species, *Palmaria palmata* and *Solieria chordalis*, were hydrolyzed with cellulase and hemicellulase (separately or in combination) under HHP (400 MPa, 20 min). The HHP-assisted enzymatic treatment improved the extraction of specific molecules such as proteins, polyphenols and polysaccharides, but their effects are highly dependent on the macroalgae species. Consequently, the antioxidant activity of extracted fractions was improved by over 2.8 times for the treatment with HHP with hemicellulase. Antioxidant activity was highly correlated with polysaccharide (89%) and protein (83%) contents for *S. chordalis,* and with polyphenol (65%) for *P. palmata*. Our experiments demonstrated, for the first time, the potential of HHP-assisted enzymatic extraction of various phytochemicals from red macroalgae and the fact that their effects are highly dependent on the macroalgae species used.

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1. Introduction

Macroalgae contain mainly carbohydrates (up to 50% of dry weight), lipids (1–5%), proteins 10–47%), minerals (8–40%), and phenolic compounds (up to 25%) although the composition might vary according to species, as well as season and location of harvest (Beaulieu et al., 2016; Cardoso et al., 2015).

As that macroalgae are exposed to large environmental fluctuations such as desiccation, temperature, salinity, light, and nutrient availability of essential nutrients. As a result, they develop adaptive and protective mechanisms producing biologically unique and active molecules with nutritional, functional and biological properties (Connan et al., 2007; Tierney Michelle et al., 2010). Therefore, they are a sustainable and attractive biomass for the formulation of human and animal foods (Fleurence, 1999b; Garcia-Vaquero and Hayes, 2016; Rioux et al., 2017). Consequently, macroalgae have been extensively studied as a potential source of bioactive compounds such as proteins and peptides, polysaccharides, lipids, and polyphenols (Abou Zeid et al., 2014; Airanthi et al., 2011; Beaulieu et al., 2015; Bondu et al., 2015; Chandini et al., 2008; Charoensiddhi et al., 2015; Harnedy et al., 2014, 2015; Heo et al., 2005; Je et al., 2009; Karmakar et al., 2010; O'Sullivan et al., 2011). More specifically, red macroalgae such as *Palmaria palmata* are consumed widely and contain high levels of nutrients. *P. palmata* contains 20, 61, 0.4, and 13% of protein, carbohydrate, lipids, and ash, respectively (Fleurence, 1999b; Wang et al., 2010). In comparison, *S. chordalis* which is an invasive species mostly used for fertilization, consists of 22, 40, 0.9, 0.9 and 25% proteins, neutral sugars, lipids, total phenol, and ash, respectively (Hardouin et al., 2014b). In addition, *S. chordalis* has been extensively researched for its bioactive polysaccharides such as carrageenans (Boulho et al., 2017; Stephanie et al., 2010). Moreover, peptides and phenolic compounds derived from these macroalgae have demonstrated *in vitro* antioxidant and ACE inhibitory activities (Bondu et al., 2015; Wang et al., 2010; Yuan et al., 2005). Despite the potential benefits of algal ingredients in human health, extracting these intracellular biomolecules is challenging due to the high degree of structural complexity and rigidity of the cell wall (Deniaud-Bouët et al., 2014). Conventional solvent-based extraction techniques have several drawbacks such as toxicity to human and environment, higher energy and time consumption, lower selectivity, and degradation of bioactive molecules during processing (Charoensiddhi et al., 2015; Kadam et al., 2013).

The use of cell wall degrading-enzymes (polysaccharidases) and proteases (EAE) could improve the extraction yield, total polyphenol content, antioxidant activities and increase protein digestibility (Fleurence, 1999a, b; Fleurence et al., 1995; Heo et al., 2005). For example, the treatment of *P. palmata* with both polysaccharidases and proteases increased the extraction yield of polyphenols and other bioactive compounds, and improved antioxidant activities (Wang et al., 2010). Although enzymatic treatment is considered milder and safer for human consumption as compared to the processes that use harsh solvents (e.g., NaOH), this technique may not be economically viable in large scale production due to the higher concentration (cost) and instability of enzymes required (Harnedy and FitzGerald, 2013). Therefore, pretreatment of macroalgal biomass during or prior to the EAE could be another viable alternative to improve the extraction yield and decrease enzyme load (Bourgougnon, 2014).

In this context, applications of non-thermal high hydrostatic pressure (HHP) and ultrahigh pressure at the range of 100–600 MPa are currently being explored for their effects on proteins and extractability of bioactive peptides and vitamins from plant-based biomass (Xi, 2017). Indeed, HHP produces structural modification (unfolding) of protein, exposing enzyme cleavage sites that are inaccessible in the native proteins. Several studies using HHP have demonstrated improved protein digestibility and bioactive peptide yield and profile, increasing the bioactivities of the hydrolysates produced (Balny and Masson, 1993; Chao et al., 2013; Garcia-Vaquero and Hayes, 2016; Girgih et al., 2015; Knudsen et al., 2002; Perreault et al., 2017). Moreover, simultaneous pressurization and enzyme treatment improved the hydrolysis of milk protein (Chicón et al., 2006). Some recent studies have shown that pressurized liquid extraction methods, which use significantly lower pressure (3–20 MPa) and higher temperature (50–200 °C) than HHP, could increase polyphenol yields and extraction of antioxidant and antibacterial components from macroalgae (Boisvert et al., 2015; Sánchez-Camargo et al., 2016; Tierney et al., 2013). In a recent review by Xi (2017), high pressure treatment is considered one of the greenest technologies with considerably shorter extraction time and higher yield compared to other conventional techniques. Moreover, the use of mild temperatures makes this process especially attractive in the extraction of thermosensitive bioactive compounds. However, to the best of our knowledge, the use of HHP on algal biomass has not yet been studied. It is important to integrate emerging and green technologies such as HHP to make better use of a highly abundant marine biomass like macroalgae.

Therefore, the aim of this study was to determine the feasibility of HHP (400 MPa–20 min) application as an assisted extraction treatment for two red macroalgae: *P. palmata* and *S. chordalis*. The biomass was simultaneously hydrolyzed using cellulase and hemicellulase, both separately and together. The efficiency of HHP treatment was analyzed by determining the total solids, polyphenol, protein and polysaccharide content of extracted fractions.

2. Materials and methods

2.1. Materials

2.1.1. Algal material

P. Palmaria was harvested from Cap-aux OS, on the Quebec coast in Gulf of St Lawrence (Canada) whereas *S. Chordalis* was harvested on the coast of Brittany (France). They were provided dried and grounded. The chemical compositions of the macroalgae are summarized in Table 1.

2.1.2. Chemicals and reagents

Cellulase, Hemicellulase, Folin and Ciocalteu's phenol reagent, anhydrous sodium carbonate, gallic acid, D-(+)-sucrose, D-(−)-fructose, D-(+)-glucose, 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH), sodium phosphate monobasic monohydrate, sodium phosphate dibasic heptahydrate, trolox (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, and fluorescein sodium salt were purchased from Sigma Aldrich (Saint-Louis, MO, USA). HCl and certified ACS Plus methanol, optima grade acetone and acetic acid were purchased from Fisher Scientific (Ottawa, ON, Canada). Finally, Tris-base and NaOH pellets were obtained from VWR International (Missisauga, ON, Canada).

Table 1
Chemical composition of the two macroalgae species.

Algae species	Humidity (%)	Protein (%)	Carbohydrates (%)	Lipids (%)	Ashes (%)
P. palmata	7.77 ± 0.12[a]	10.20 ± 0.1	60.13 ± 0.24	0.80 ± 0.07	21.10 ± 0.47
S. chordalis	9.62 ± 0.19	10.12 ± 0.15	36.38 ± .39	1.00 ± 0.21	42.88 ± 0.64

[a] Mean values of three replicates ± standard deviation.

2.2. Method

2.2.1. Experimental design

Ground *P. palmata* and *S. chordalis* (6% w/v) were hydrated overnight at 4 °C in Tris-HCl buffer at pH 5.0 and stirred with a magnetic stirrer. The enzymatic hydrolysis was performed under HHP process or at atmospheric pressure, using four different enzymatic treatments, as shown in Table 2. The pH and temperature of the hydrated samples were adjusted to the optimal values for the enzymes (pH 5.0 and 37 °C for cellulase and pH 4.5 and 40 °C for hemicellulase). Enzymes were added at an E/S ratio of 5:100 and samples were immediately pressure-treated at 400 MPa for 20 min (Mini Foodlab FPG5620, Stansted Fluid Power Inc. Essex, UK). The 400 MPa pressure was chosen since it was found to partially modify plant cellulose structure, allowing greater accessibility of enzymes without impacting the enzyme activity (Oliveira et al., 2012). At a pressurization rate of 50 MPa/min, it took 8 min to reach 400 MPa. The enzymatic hydrolysis at atmospheric pressure (control) was performed for 28 min. Control samples included treatments at atmospheric pressure as well as the extraction without enzymes. After the hydrolysis step, the pH was adjusted to 10 with NaOH (0.5 M) to inhibit enzyme activity.

2.2.2. Calculations and analyses

2.2.2.1. Extraction yield. The enzyme- and pressure-treated solutions were centrifuged at 8000 × *g* for 30 min. The supernatants were next separated from the residues and the pH was adjusted to 7.0. Finally, the supernatants, containing soluble materials, were recovered and freeze-dried. The extraction yield was then calculated using equation (1)

$$Y\,(\%) = \frac{m_R}{m_I} \times 100 \quad (1)$$

where, Y is the extraction yield (expressed in %), m_R is the quantity of dry matter recovered after freeze drying (g) and m_I is the initial algal mass (g). The freeze dried samples were used to determine protein, polysaccharides, polyphenols contents, and antioxidant activity (ORAC). The total protein, polyphenol and polysaccharide contents were multiplied by the extraction yield to determine the relative yield of each component on the basis of initial algal mass.

2.2.2.2. Protein content. The protein content in *P. palmata* and *S. chordalis* supernatants obtained after centrifugation was determined in duplicate by the Dumas combustion method (Truspec, LECO Corporation, St. Joseph, MI, USA). A conversion factor of 4.92 was used to convert percentage nitrogen to protein content (Simonne, 1997).

2.2.2.3. Total polyphenol content. Total polyphenol content was determined using the Folin and Ciocalteu method (Agbor et al., 2014) with modifications. Briefly, 20 µL of *P. palmata* and *S. chordalis* supernatant samples recovered after enzymatic treatment at a concentration of 25 mg/mL or a standard solution (gallic acid at 50, 100, 250 and 500 mg/L) were transferred into a 96 well microplate. Next, 100 µL of diluted (1/10) Folin and Ciocalteu reagent was added. After 4 min, 80 µL of a 7.5% solution of Na_2CO_3 was added to inactivate the Folin and Ciocalteu reagent. The microplate was shaken and incubated in the microplate reader for 1 h before reading the absorbance at 765 nm with a xMark Microplate spectrophotometer (Bio-Rad, Mississauga, ON, Canada). Total polyphenol content was expressed as mg/L of gallic acid equivalent.

2.2.2.4. Polysaccharide content. The polysaccharide content of the algal samples in the supernatants, after enzymatic treatments, was determined using a Waters HPLC system (Millipore Corp., Milford, MA. USA) including a refractive index detector (Hitachi model L-7490). Samples were prepared at a concentration of 10 mg/mL, vortexed and filtered through a 0.45 µm nylon filter into an amber vial. A sample volume of 50 µL was injected onto the column (Waters Sugar Pak-I, 6.5 × 300 mm, Waters). The column was maintained at 90 °C. The isocratic mobile phase consisted of a solution of EDTA (50 mg/mL) and had a flow rate of 0.5 mL/min. The run time was 30 min.

2.2.2.5. Antioxidant activity. Oxygen radical absorbance capacity (ORAC) assays were performed in triplicate according to Perreault et al. (2017) on both freeze-dried macroalgal supernatants with a Fluostar Galaxy fluorometer (BMG LabTech, Durham, NC). Algal supernatants (50 mg) were dissolved in a 10 mL solution of acetone/water/acetic acid (AWA) in proportions of 70/29.5/0.5% by vortexing for 30 s. Afterwards, the solutions were sonicated for 5 min at 37 °C with periodic shaking and cooled down to room temperature for 10 min. Then, the samples were centrifuged at 4000 rpm for 15 min. The supernatant was collected and the pellet was washed again with 10 mL of AWA solution. The supernatants were pooled and diluted with AWA solution to obtain a final volume of 250 mL. Trolox control standards (6.25, 12.5, 25 and 50 µM Trolox) were used. The results obtained were expressed as micromoles of Trolox equivalent (TE) per gram of freeze-dried sample (µmol TE/g), and were multiplied by the extraction yield and represented as µmol TE/g of initial mass.

2.2.2.6. Statistical analyses. Statistical analyses were performed using Statistical Analysis System (SAS) University Edition, SAS® Studio 3.5 software. Wisest orthogonal contrasts and planned comparisons ($\alpha = 0.05$) were performed. A-factorial ($2 \times 2 \times 2$ – HHP × Cellulase × Hemicellulase) complete block ($n = 3$) design was used for each macroalgae. In addition, the Pearson coefficient was calculated to determine the highest correlation between antioxidant activity and extraction of protein, polyphenols and polysaccharides. For each experiment, the experimental unit was a sample which received one treatment.

3. Results and discussion

3.1. Effect on extraction yield

The efficiency of enzymatic treatment under HHP was evaluated on total solid extraction yield in the soluble fraction (supernatant) and compared with two control samples: 1) without enzymatic hydrolysis at atmospheric pressure and 2) with enzymatic hydrolysis at atmospheric pressure. For both types of algae, one simple effect of the enzyme (cellulase – $p = 0.0019$ and $p = 0.0274$, and hemicellulase – $p = 0.0107$ and 0.0467, respectively, for *P. palmata* and *S. chordalis*) was found to improve the extraction yield. Nevertheless, the results for *P. palmata* (Table 3) show that only enzymatic treatment by cellulase combined with hemicellulase significantly improved the total solids extraction yield compared to extractions performed without enzymes. The application of cell wall degrading enzymes increased the extraction yield from 9 to 37% for *P. palmata*. However, the application of HHP, with or without enzymes, had no effect on total solid extraction.

For *S. chordalis*, both enzymes demonstrated a simple positive effect, whereas HHP did not have an effect on total solid extraction yield and

Table 2
Factorial design (2*2*2) with HHP treatment, cellulase (C) and hemicellulase (H).

Enzymes		HHP	
Cellulase (C)	Hemicellulase (H)	*0*[a]	*1*
0	*0*	0 0 0	1 0 0
	1	0 0 1	1 0 1
1	*0*	0 1 0	1 1 0
	1	0 1 1	1 1 1

[a] 0: without and 1: with.

Table 3
The effect of enzymatic and HHP treatments on extraction yield (%).

Cellulase (C)	Hemicellulase (H)	*Palmaria palmata*		*Solieria chordalis*	
		HHP		HHP	
		0	1	0	1
0	0	50.76 ± 3.13^{b*}	51.11 ± 2.87^{b}	26.09 ± 1.30^{a}	29.38 ± 1.26^{a}
0	1	55.29 ± 8.27^{a,b}	62.41 ± 5.01^{a,b}	29.22 ± 6.52^{a}	34.24 ± 4.54^{a}
1	0	60.36 ± 5.52^{a,b}	61.57 ± 6.20^{a,b}	32.14 ± 2.04^{a}	32.16 ± 1.33^{a}
1	1	69.69 ± 0.5^{a}	66.28 ± 11.82^{a}	34.69 ± 5.12^{a}	34.87 ± 3.8^{a}

*Mean of three replicates ± standard deviation.
Mean values for a microalgal species with different letters are significantly different ($P < 0.05$).
A "0" value means absence of enzyme or HHP treatment.
A "1" value means that enzyme and/or HHP were used or applied.

no differences between the treatments were observed ($p = 0.8026$). Additionally, hemicellulase treatment under HHP improved total extraction yield by 13 and 17% for *P. palmata* and *S. chordalis*, respectively.

Concerning the impact of enzymes, our results agree with those of Hardouin et al. (2014b) and Wang et al. (2010) who observed improvements in dry matter yield of tested algae, including *S. chordalis* and *P. palmata*, using several carbohydrases. They also measured extraction yields in a similar range with cellulase for *P. palmata* (66%) and xylanase for *S. chordalis* (22%) (Hardouin et al., 2014b; Wang et al., 2010). The slight differences observed between our study and those in the literature could be due to differences in experimental parameters, including time (28 min vs. 5 or 24 h) and chemical composition of initial biomass used (particularly carbohydrates and protein). Moreover, a clear distinction was also observed in the extraction yield between the two algal species – *P. palmata* had twice the extraction yield of *S. chordalis*. This difference may be associated with differences in chemical composition, for example, the carbohydrate and ash contents (Table 1) and with differences in the cell wall structures of the two macroalgae (Fleurence, 1999b). Therefore, we demonstrated that the application of HHP had no effect on the total extract yield for both macroalgae species.

3.2. Effect on phytochemical extraction and bioactivity

3.2.1. Protein extraction

The efficiency of HHP and enzymatic treatment on protein extraction was analyzed based on the amount of protein content in the soluble fraction (supernatant) of each treatment. The effects of HHP combined with several enzymatic treatments on protein extraction for *P. palmata* and S. *chordalis* are shown in Fig. 1. Statistical calculations for *P. palmata* highlight a three-way interaction between parameters (HHP*C*H), as shown in Table 4. All major effects and interactions observed on all phytochemicals studied are summarized in Table 4 and discussed where appropriate in the text.

The use of HHP led to different results for proteins extracted from *P. palmata* (Fig. 1a) without additional enzyme or with either cellulase and/or hemicellulase. The HHP treatment did not improve the extraction when no enzyme was used. However, the application of high pressure increased protein extraction by cellulase or hemicellulose, alone, but not when both enzymes were used with HHP.

In the case of *S. chordalis* (Fig. 1b), HHP treatment increased protein extraction when treated without enzymes and when treated with hemicellulase. Conversely, combining HHP and cellulase (Table 4) inhibited the positive effect of HHP, as no differences were observed between control and pressure-treated protein extraction using cellulase or combined cellulase and hemicellulase. The use of cellulase enzymes increased protein extraction slightly from 30 to 34 mg/g of initial weight. The HHP treatment alone increased the protein yield by 8.4% while HHP combined with cellulase increased the protein content by 17%. Hemicellulase alone had no effect but when combined with HHP increased the protein content to close to that of HHP and cellulase treatment. Lastly, cellulase and hemicellulase increased protein extraction but HHP had no effect. The results shown here agree with previous observations (Fleurence et al., 1995; Hardouin et al., 2014b; Wang et al., 2010) which showed a significant increase in protein extraction from *P. palmata* and *S. chordalis* treated with cellulase alone. In contrast, when *P. palmata* was treated with cellulase and xylanase (hemicellulase) simultaneously, the extraction yield did not improve and was close to that of the control (no enzyme) (Fleurence et al., 1995). In accordance with the observations of Oliveira et al. (2012), our results show that HHP treatment is efficient in disrupting and disintegrating cell wall structure to increase accessibility of enzymes to the substrate like cellulose and, thus, releasing proteins (Oliveira et al., 2012). However, the efficiency is largely dependent on the type of enzyme as well as on the cell structure and composition of macroalgae species (Fleurence, 1999a).

3.2.2. Total polyphenols extraction

The effects of HHP and/or enzyme treatments on the extraction of total polyphenols are shown in Fig. 2. For both types of macroalgae, HHP treatment (Table 4) and cellulase had significant effects on total polyphenol extraction but there was no relationship between these two parameters. For *P. palmata*, regardless of the pressure applied, treatments using cellulase or cellulase combined with hemicellulase increased the total polyphenol extraction by 36–58% compared to the treatment with no enzyme. In contrast, hemicellulase alone did not improve polyphenol extraction. Similarly, for *S. chordalis*, (Fig. 2b), cellulase alone or in combination with hemicellulase significantly improved polyphenol extraction compared to samples without enzyme, whether HHP treatment was used or not ($P = 0.0001$). Our results suggest that enzymes like cellulase and hemicellulase disrupted or weakened the structural integrity of the seaweed cell wall and HHP

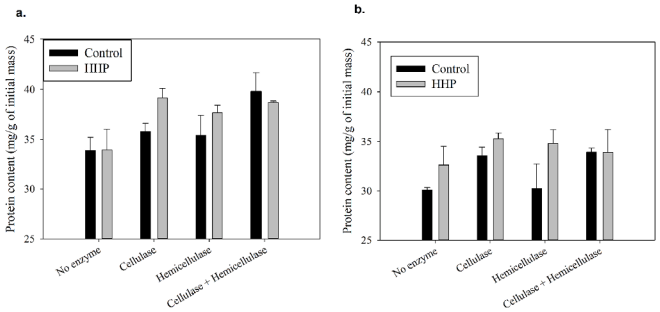

Fig. 1. Effect of HHP and enzymes on protein extraction from *P. palmata* (a) and *S. chordalis* (b). Data are mean values of three replicates ± standard deviation.

Table 4
Significant differences observed for each phytochemical and antioxidant activity (independent variable) studied by algal species.

Independent variable	*Palmaria palmata*		*Solieria chordalis*	
	Significant parameters	P value	Significant parameters	P value
Proteins	HHP × C × H[a]	0.0101	HHP × C	0.0453
Polyphenols	C	< 0.0001	C	0.0001
	HHP	0.0241	HHP	0.015
Polysaccharides	H	0.0004	C × H	0.0275
	C	0.0164	HHP × C	0.0001
	HHP	0.0045		
	C × H	0.0009	C × H	0.0178
ORAC	HHP × H	< 0.0001		
	HHP × C	0.0052	HHP × C	0.0481

[a] HHP: High hydrostatic pressure; C: Cellulase; H: Hemicellulase.

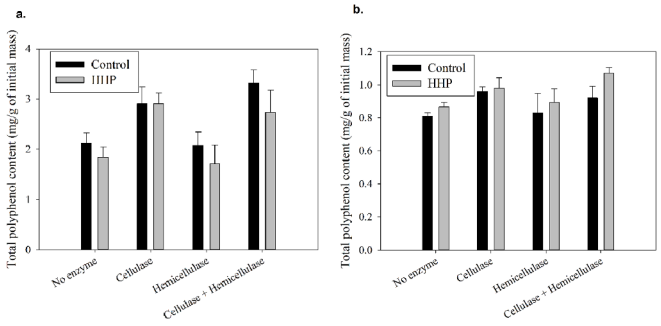

Fig. 2. Effect of HHP and enzyme on total polyphenol extraction from *P. palmata* (a) and *S. chordalis* (b). Data are mean values of three replicates ± standard deviation.

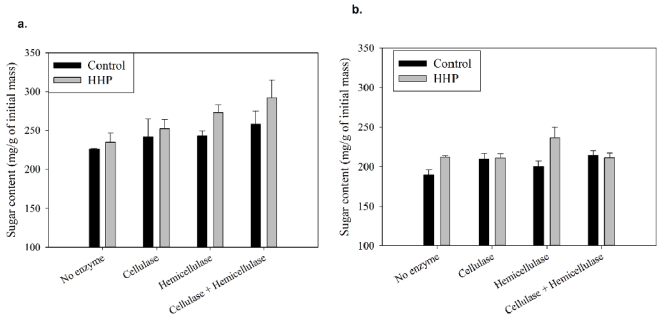

Fig. 3. Effect of HHP and enzymes on polysaccharide extraction from *P. palmata* (a) and *S. chordalis* (b). Data are mean values of three replicates ± standard deviation.

increased the accessibility of enzymes, accelerating the release of intracellular polyphenols. Specific differences in the effects of applied treatments (HHP and enzymes) on the two macroalgae species may be due to the structural and chemical compositional differences discussed earlier. The total polyphenol content was higher in *P. palmata*, with > 2 mg/g of initial mass, than in *S. chordalis*, with < 1 mg/g of initial mass. In contrast to Wang et al. (2010) and Hardouin et al. (2014b), our study showed a significant improvement in total polyphenol extraction for both types of macroalgae.

3.2.3. Polysaccharide extraction

The extraction of soluble polysaccharides using HHP treatment and polysaccharidase enzymes is shown in Fig. 3. For *P. palmata*, HHP and individual enzyme treatments had significant effects on polysaccharide extraction. The HHP treatment slightly increased polysaccharide extraction for all enzyme treatments (with or without cellulase and/or

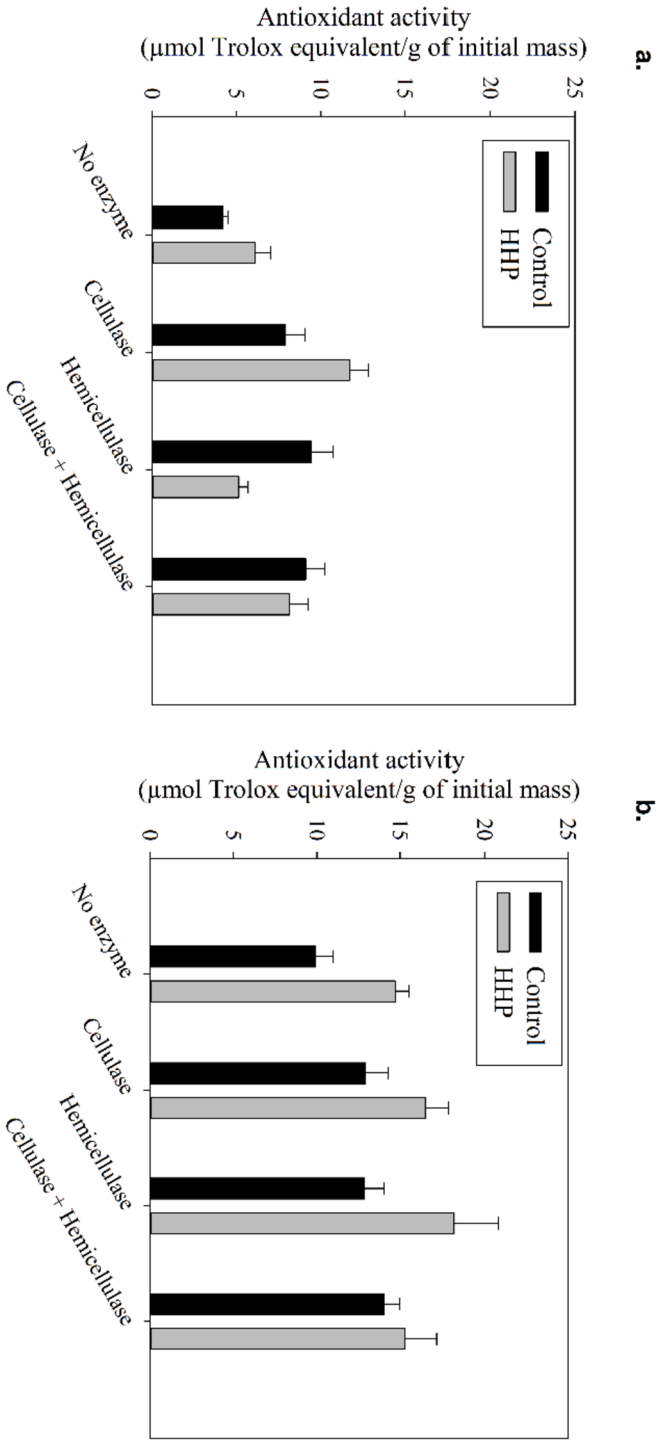

Fig. 4. Effect of HHP and enzymes on antioxidant activity of *P. palmata* (a) and *S. chordalis* (b). Data are mean values of three replicates ± standard deviation.

hemicellulase). However, unlike for protein and polyphenol, hemicellulase improved the polysaccharide extraction from 225 (without enzymes) to 243 mg/g initial mass. Moreover, HHP coupled with hemicellulase enhanced the extraction of polysaccharides up to 273 mg/g of initial mass. Similarly, a synergistic effect was observed for the treatment containing cellulase and hemicellulose, producing the highest polysaccharide content (291 mg/g of initial mass) with HHP.

Compared to *P. palmata*, the yields of extracted polysaccharides were lower for *S. chordalis* (Fig. 4b) which could be due to the differences in their carbohydrate compositions (as described in section 2.1).

Table 5
Pearson correlation coefficient between ORAC and phytochemicals.

Algae species	Protein	Polyphenol	Polysaccharides
P. palmata	0.603[a]	0.651	0.203
S. chordalis	0.829	0.467	0.891

[a] A Pearson coefficient of 1 corresponds to 100% correlation.

Broadly, when performed at atmospheric pressure, the extraction was significantly improved by the use of enzymes regardless of the type of enzyme (cellulase, hemicellulase or both). Under HHP, only hemicellulase or the absence enzymes achieved higher polysaccharide yields, whereas the use of cellulase or even cellulase and hemicellulase under HHP did not improve the extraction, as yields were similar to treatment without enzymes and under HHP. Consequently, the effect of HHP depended on the presence of cellulase, confirming the relationship between these two parameters ($p = 0.0001$, Table 4). Nevertheless, treatment using hemicellulase and HHP extracted the greatest amount of polysaccharide, 24% higher than treatment without enzymes and HHP for *S. chordalis*. Unlike the study of Hardouin et al. (2014b) who observed a considerable increase in sugar extraction for *S. chordalis* using cellulase and xylanase, lower extraction efficiency of the enzymes in the present study could be explained by differences in the experimental parameters, as mentioned earlier.

3.2.4. Antioxidant activity

To evaluate the effects of enzyme and HHP treatments on the extraction of antioxidant molecules, the ORAC test was performed on the extracted samples, as shown in Fig. 4. Our *P. palmata* results show that in the absence of HHP treatment, enzymatic hydrolysis alone increases the antioxidant activity, irrespective of the type of enzyme or their combination ($p = 0.0009$). Most importantly, hemicellulase treatment increased the ORAC activity the most – over 2-fold greater than control without enzymes. In addition, HHP treatment significantly enhanced the ORAC activity of samples containing cellulase ($p = 0.0218$), whereas it decreased when hemicellulase was used either alone ($p = 0.0004$) or combined ($p = 0.2517$). The combination of cellulase and HHP treatment produced the best ORAC activity, increasing antioxidant capacity by more than 2.8 times compared to control without enzymes and pressure (from 4.20 to 11.72 μg Trolox equivalent/g of initial biomass) (Fig. 4a). Conversely, hemicellulase alone gave rise to the highest activity, while HHP reduced the activity considerably when coupled to the enzyme treatment. In previous studies, the antioxidant (ORAC) activity was associated with the polyphenol content in macroalgae, including that of *P. palmata* (Hardouin et al., 2014a; Lahaye and Vigouroux, 1992; Wang et al., 2009, 2010; Yuan et al., 2005) as well as the breakdown products of polysaccharides and proteins (Siriwardhana et al., 2008). Therefore, in order to determine the relationship between ORAC and extracted phytochemicals, Pearson's coefficient of correlation was determined for each phytochemical extracted (Table 5). For *P. palmata*, ORAC activity is mainly associated with total polyphenols (correlation coefficient of 65%), followed by proteins (60%), and finally polysaccharides (20%), as previously observed by other authors (Wang et al., 2010).

Similarly, for *S. chordalis*, ORAC activity increased with enzymatic degradation regardless of the type of enzyme used (Fig. 4b). The interaction between HHP and cellulase (Table 4) showed that HHP had a considerable effect on the antioxidant capacity only in the absence of cellulase. The highest ORAC activity was observed for samples treated with HHP and hemicellulase, which were approximately 2-fold greater than samples without enzyme and pressure treatment. The ORAC activity for *S. chordalis* was mostly associated with polysaccharides and proteins with coefficients of correlation of 89 and 83% (Table 5).

This work provides the first insights on HHP-assisted extraction of phytochemicals from algal biomass. Even though HHP had no clear effect in overall extraction yield, its application improves the accessibility of enzymes and enhances the extraction of proteins and polysaccharides for both species tested. More specifically, the use of HHP-assisted enzymatic hydrolysis improved the extraction of proteins when combined with cellulase and improved carbohydrate extraction when using hemicellulase. The cellulase enzyme (with or without HHP) was found to significantly improve the extraction of polyphenols from *P. palmata* but not from *S. chordalis*. In general, cellulase and hemicellulase combined did not have a clear effect on the extraction of any of the phytochemicals studied. Our results showed that both polysaccharidases and HHP treatments can improve extraction of specific molecules from red macroalgae, but their effects depend on the type of algal biomass treated, probably due to differences in chemical composition and cell wall structure. The improvement in antioxidant activity correlated strongly with polyphenol extraction for *P. palmata,* and with polysaccharide and protein extractions for *S. chordalis*. Therefore, the HHP process must be optimized for each species of seaweed. Going forward, studying both the effects of HHP on these enzymes, characterization of their sensitivity to high pressure, and the effects of HHP pretreatment on macroalgae prior to enzymatic hydrolysis should provide more insight into best choices for HHP parameters.

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