Journal of Experimental Marine Biology and Ecology 399 (2011) 167–172

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

Distribution and radical scavenging activity of phenols in *Ascophyllum nodosum* (Phaeophyceae)

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A R T I C L E I N F O

Article history:
Received 3 October 2010
Received in revised form 20 December 2010
Accepted 4 January 2011

Keywords:
Ascophyllum nodosum
NMR
Phenol purification
Phlorotannins
Radical scavenging

A B S T R A C T

Phlorotannins have been purified and fractionated in the brown alga *Ascophyllum nodosum* using successively differential extraction, liquid–liquid separation and dialysis. Both the phenol content and the radical scavenging capacity of the resulting fractions were assayed by the Folin–Ciocalteu test and the DPPH method, respectively, whilst purity of the fractions was assessed by ^{1}H NMR analysis. The purification process resulted in the isolation of six fractions from each crude extract with only minor losses. High levels of phenols, up to 97–99%, were measured in semi-purified fractions containing phlorotannins more than 50 kDa in average molecular size, accounting for more than 95% of the ethyl acetate phenol pool. As a consequence, purity decreased in ethyl acetate fractions together with the molecular size of compounds. The importance of differential extraction based on the polarity of phenols is highlighted by the fact that most of these compounds were found in the ethyl acetate fraction after the first extraction step in 100% methanol, whilst two thirds of phenols extracted by 50% methanol remained in the aqueous phase. The radical scavenging activity of the fractions was correlated with the phenol content and was maximal in complete ethyl acetate fractions and in dialysis concentrates containing molecules more than 50 kDa in size. The specific activity of phenols was found to be maximal for molecules smaller than 2 kDa when isolated from the 100% methanol extract and 1–4 times smaller in the water phase separated from the same extract. The distribution of radical-scavenging potentials in the phenol pool of *A. nodosum* supports the idea that physiological roles and putative uses of phlorotannins are under the control of a polarity–molecular size complex.
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1. Introduction

Brown algae (Phaeophyceae) produce polymers of phloroglucinol (1,3,5-trihydroxybenzene) referred to as phlorotannins which accumulate up to 25–30% DW (e.g. Ragan and Glombitza, 1986; Targett et al., 1995). Their molecular size ranges between 162 Da (phloroglucinol) and 650 kDa (Ragan and Glombitza, 1986). Generally, phlorotannins are enclosed in vesicles called physodes which are more numerous in cells of meristematic and cortical areas (Ragan and Glombitza, 1986; Toth and Pavia, 2002). The highest levels of phenolic compounds in brown algae are found either in meristematic (Tugwell and Branch, 1989; Cronin and Hay, 1996; Van Alstyne et al., 1999) or in reproductive (Steinberg, 1984; Tuomi et al., 1989; Cronin and Hay, 1996; Van Alstyne et al., 1999) regions of the thallus.

During the early stages of development in Fucales, phlorotannins are involved in both the formation of the cell wall (Schoenwaelder and Wiencke, 2000) and the elaboration of adhesive cement (Vreeland et al., 1998). Their role in defense mechanisms against grazers (Pavia and Toth, 2000; Sotka et al., 2002), pathogens (Ragan and Glombitza, 1986) and epiphytes (Jennings and Steinberg, 1997; Steinberg et al., 1998) has been largely documented. They seem to be very efficient in photoprotection and particularly against UV radiation (Pavia et al., 1997), whereas the contribution of carotenoids, terpenoids or other protective molecules has not been yet concomitantly evaluated (Connan et al., 2004). Their effectiveness as anti-stress compounds depends essentially on their antioxidant/radical scavenging activity and is related to their levels in seaweed extracts (Jimenez-Escrig et al., 2001).

Many studies have reported both the isolation by chemical ways and the structural characterization of numerous oligomers (e.g. Ragan and Glombitza, 1986; Glombitza and Pauli, 2003) and polymers (McInnes et al., 1984) of phlorotannins, but usually without any biological activity assessment. However, although most of the activity studies have been carried out on crude extracts, some works have suggested that activity of phenols in Phaeophyceae seems to be related to their structure and especially to their degree of polymerisation, with oligophenols generally considered more active than highly polymerised compounds (e.g. Nakamura et al., 1996). Globally, few studies have dealt so far on the specific roles of phlorotannin fractions. Geiselman and McConnell (1981) studied the effect of size

Abbreviations: Da, Dalton; DPPH, 2,2-diphenyl-1-picrylhydrazyl; NMR, Nuclear Magnetic Resonance; ppm, parts per million; UV, Ultraviolet radiation.

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doi:10.1016/j.jembe.2011.01.002

fractions between smaller than 30 kDa and more than 300 kDa in the protection of brown seaweeds against grazing by snails. Barwell et al. (1989) showed that polyphenols of four species of Fucaceae between 30 and 100 kDa were able to inhibit the activity of mammalian digestive enzymes, but the purification process of fractions was rather basic. The distribution of molecular size fractions in four species of Fucaceae and their effect has been tested on the assimilation of *Ulva* by grazing fishes (Boettcher and Targett, 1993). Arnold and Targett (1998) quantified in situ rates of phlorotannin synthesis and separated molecules below and above 30 kDa on Amicon membranes, but they did not measure activities. Kubanek et al. (2004) isolated and purified phlorotannins from *Fucus vesiculosus* using three solvent phases differing by their polarity, *i.e.* dichloromethane, water and ethyl acetate. They assayed polarity fractions for their deterrent action against grazers, but not the phenol size fractions isolated by ultrafiltration from both the water and ethyl acetate phases. Cérantola et al. (2006) purified poly-fucols and poly-fucophlorethols and dosed their anti-oxidant activities in hydro-alcoholic extracts of *Fucus spiralis*.

In this work, we describe an experimental process to purify phenol fractions from *Ascophyllum nodosum* based on both the polarity and the molecular size of phlorotannins. The radical scavenging potential of these fractions has been quantified and results are discussed relatively to both the purity and the apparent specific activity of phenols.

2. Materials and methods

2.1. Plant material and preparation of the fractions

Thalli of *A. nodosum* (Le Jolis) were collected in October 2005 in the intertidal zone at Saint-Anne du Portzic, on the western coast of Brittany (France). Only the lower third of each thallus, *i.e.* between 30 and 70 cm over 1–2 m total length, was cut off since this area is few epiphyted and wounded and no significant difference in phenol contents has been reported along plants of *Ascophyllum* (Connan et al., 2006). Typically, ca. 50 basal fragments were washed thoroughly with filtered seawater and then with de-ionised water, in order to remove residual sediments and salts. Algae were then surface dried with paper towel, weighed up to 1 kg fresh weight, chopped into small pieces and homogenized in the extraction solvent using a Waring Blender. Phenolic compounds were first extracted with 5 L of pure methanol for 3 h at 40 °C under orbital stirring in the dark. After extraction, the mixture was centrifuged at 4500 *g* for 15 min at 4 °C in an Eppendorf 5810 R centrifuge (Eppendorf A.G., Hamburg, Germany). The supernatant was filtered on cotton wool and then concentrated to dryness under reduced pressure at 40 °C using a rotary evaporator. About 50 mL of de-ionised water was then added to the residue to give the first crude extract (Cr1). The pellet of centrifugation containing residual tissues was re-extracted with aqueous methanol (50%, v/v) under the same conditions. The second crude extract (Cr2) was obtained after concentration of the second supernatant and complete removal of methanol. Cr1 was then successively treated with 4 volumes of hexane, 4 volumes of dichloromethane and 5 volumes of ethyl acetate. Cr2 was treated with 1 volume of hexane, 2 volumes of dichloromethane and 6 volumes of ethyl acetate. Polyphenolic compounds present in ethyl acetate fractions were subjected to successive dialysis steps on MWCO (molecular weight cutting opening) 50 kDa and MWCO 2 kDa membranes (Spectra/Por dialysis tubing, Spectrum Europe, Breda, The Netherlands) against de-ionised water, for 5 days at 4 °C in the dark. All results were expressed on a dry weight (DW) basis by excising about 15 g fresh tissues from the thalli submitted to extraction and drying in an aerated oven at 60 °C for 3 days. DW of *Ascophyllum* tissues was 28.52 ± 1.44% of the initial fresh weight. Crude extracts and subsequent fractions were freeze-dried before re-using in a Christ Beta 1–8 LD system (Christ, Osterode am Harz, Germany). The main steps of the fractionating process are described in Fig. 1.

2.2. NMR studies

The global structural composition of the crude extracts and of their subsequent fractions was assessed by means of ^{1}H NMR analyses on a Bruker Avance 400 and/or on a Avance 500 spectrometer equipped with a $^{1}H/^{13}C/^{15}N$ TCI cryoprobe, using standard pulse sequences available in the Bruker software (Bruker, Wissembourg, France). All spectra were recorded in D_2O, at 298 °K. Chemical shifts were expressed in ppm relative to 2,2,3,3-tetradeuterio-3-(trimethylsilyl)-propanoic acid sodium salt as external reference.

2.3. Quantification of phenols

Phenols were assayed by the Folin–Ciocalteu method adapted from Sanoner et al. (1999). Absorbance was read at 700 nm on a UV 160A spectrophotometer (Shimadzu, Croissy, France) with phloroglucinol (1,3,5-trihydroxybenzene, Sigma, Saint Quentin Fallavier, France) as standard.

2.4. DPPH free-radical scavenging assay

The radical scavenging activities of the various extracts were assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free-radical method (adapted from Saint-Cricq de Gaulejac et al., 1999), a technique easily developed and applied in routine both in the laboratory and at the industrial level (Connan et al., 2004, 2006, 2007). Aliquots of 300 μL of fractions were added to 3 mL of DPPH solution [3×10^{-4} mol L^{-1} in methanol/water mixture (90/10)]. After standing for 60 min, the optical density of the samples was read at 517 nm against de-ionized water. Absorbance was then transformed into a percentage of inhibition versus de-ionized water. To assess the relationship between the content and the activity of phenols in the various fractions, a AI50 index was calculated, corresponding to the amount of either the fraction as a mass in μg (AI50 F) or of the phenols contained in the fraction (AI50 PC) necessary to obtain 50% of inhibition in the DPPH assay (cf. Connan et al., 2004). To estimate the radical scavenging potential of each fraction, an Activity Potential Index (API) was calculated as in the following equation:

$$API = 1 \ / \ AI50\ PC\ ^{*}\ m\ PC$$

in which m PC is the average mass of phenols in the fraction expressed in mg and API has no dimension.

2.5. Statistical analysis

Statistical analysis was performed using Statistica 8 software. After checking the homoscedasticity of variances, the differences between data were tested either by ANOVA or by Kruskal–Wallis tests. When data showed significant differences ($p<0.05$), an *a posteriori* test was used to rank samples according to either their purity or radical-scavenging activity, LSD and box-and-whisker analysis, respectively. A Pearson's test was used to assess putative correlations between the activity and the quantity of phenolic compounds in the fractions.

3. Results

3.1. Phenol contents

Phenol contents differed significantly between fractions (ANOVA, log transformed variable, $F=345.68$, $p<0.00001$). Phenol contents in crude extracts 1 (Cr1) obtained in pure methanol were significantly

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