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Comprehensive chlorophyll composition in the main edible seaweeds

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ABSTRACT

Natural chlorophylls present in seaweeds have been studied regarding their biological activities and health benefit effects. However, detailed studies regarding characterization of the complete chlorophyll profile either qualitatively and quantitatively are scarce. This work deals with the comprehensive spectrometric study of the chlorophyll derivatives present in the five main coloured edible seaweeds. The novel complete MS^2 characterization of five chlorophyll derivatives: chlorophyll c_2, chlorophyll c_1, purpurin-18 *a*, pheophytin *d* and phytyl-purpurin-18 *a* has allowed to obtain fragmentation patterns associated with their different structural features. New chlorophyll derivatives have been identified and quantified by first time in red, green and brown seaweeds, including some oxidative structures. Quantitative data of the chlorophyll content comes to achieve significant information for food composition databases in bioactive compounds.

1. Introduction

The seaweed industry provides a wide variety of products that have an estimated total annual value of US$ 5.5–6 billion, and *ca.* 90% of the production comprises food products for human consumption. They form part of the traditional dietary habits in Asian countries, mainly Japan, China and Korea but recently, seaweeds have drawn the interest of food industry in Western countries because of their nutritional value and as raw materials for supply of components in functional foods (Shahidi, 2009). They are excellent sources of polysaccharides, with relatively high protein content, including all of the essential amino acids, a remarkable presence of essential fatty acids with a nutritionally ideal n-6/n-3 fatty acid ratio, minerals, vitamins and trace elements. Edible seaweeds are taxonomically grouped into three different groups depending on their thallus colour, brown (Ochrophyta), red (Rhodophyta) and green (Chlorophyta). The three most important seaweeds used for human consumption (FAO, 2003) are species from *Porphyra* (common Japanese name, Nori), *Laminaria* (Kombu) and *Undaria* (Wakame). Among the green seaweeds, it is highly appreciated *Enteromorpha* (Aonori) and *Ulva* (Sea Lettuce).

Several kinds of secondary metabolites with potential benefits to human health have been described in seaweeds (Pangestuti & Kim, 2011). For example, it has been shown that the administration of several green seaweeds increased the fecal excretion of dioxins due to its chlorophyllic composition, and consequently the ingestion of green seaweeds can be consider as a new approach in the treatments of patients exposed to lipophilic xenobiotics. Specifically, the pheophytin *a* from *Porphyra* and *Enteromorpha* species has been identified as a potent suppressor against genotoxin-induced *umu* C gene expression in *S. typhimurium* (TA 1535/pSK 1002) probably associated with carcinogenesis (Okai, Okai, Yano, & Otani, 1996). According to this, pheophytin *a* derivatives have been proposed to display a potent suppressive activity against chemically induced mouse skin tumorigenesis. Pheophorbide *a* from brown seaweeds has been also proposed as a candidate for treating neurodegenerative diseases such as Alzheimer's disease (Ina, Hayashi, Nozaki, & Kamei, 2007) due to its capacity to promote the differentiation of PC12 cells. Occasionally, consumers have shown allergic reactions after ingestion (Hwang et al., 2005), and pheophorbide *a* has been pointed as the hypothetic responsible compound. Consequently, different chlorophyll derivatives isolated from seaweeds have attracted considerable attention in the fields of food, cosmetic and pharmacology (Pangestuti & Kim, 2011).

A difference with microalgae, whose detailed chlorophyll composition is known (Garrido, Airs, Rodríguez, Van Heukelem, & Zapata, 2011; Garrido, & Zapata, 1996), the "recalcitrant" extracellular material of seaweeds (macroalgae) has made difficult its complete characterization. Analysis of chlorophyll compounds in

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seaweeds has been restricted mainly to presence of chlorophyll *a*, chlorophyll *b* (Lin et al., 2011), chlorophyll *c* (Fujii et al., 2012) and pheophorbide *a* (Amorim, Lage-Yusty, & López-Hernández, 2012; Ferraces-Casais, Lage-Yusty, Rodríguez-Bernaldo de Quirós, & López-Hernández, 2012; Hwang et al., 2005).

The present work deals with the detailed and complete analysis of the chlorophyll fraction of the five seaweeds more consumed in the world. The identification is made by HPLC-hrTOF-MS, using different sources, ESI or APCI, in function of the polarity of compounds. Considering the biological activities and health benefit effects of the different chlorophyll catabolites and the high intake levels of seaweeds, it is essential to know exactly the composition of these sea foods.

2. Materials and methods

2.1. Raw material

Green seaweeds, Aonori (*Enteromorpha* spp.) and Sea Lettuce (*Ulva* spp.), were provided by Suralgae (Cádiz, Spain). Nori (*Porphyra umbilicalis*), Wakame (*Undaria pinnatifida*) and Kombu (*Laminaria ochroleuca*) were provided by Algamar (Pontevedra, Spain). The five seaweed species were collected on the Atlantic littoral region on the south western part (Cádiz) and the north western part (Pontevedra) of Spain. The dried material (25–45 °C for 30–45 h) is supplied in vacuum sealed bags. *Acaryochloris marina* NBRC 102967 for chlorophyll *d* identification was supplied by the Biological Resource Center of the National Institute of Technology and Evaluation (Chiba, Japan). The culture procedure for *Acaryochloris marina* was adopted from Swingley et al. (2008). Cultures were shaken at 125 rpm under a photon irradiance of 20 mol photons/m^2 s of light at 30 °C during four weeks. Cultures were grown as 10 mL in 50-mL Erlenmeyer flasks. The used media (MGB-11) was supplemented with 20 mM N-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid (TES) adjusted to pH 8.35. The medium was changed every one or two days.

2.2. Reagents

Selenium dioxide (sublimed for synthesis, 98%), ammonium sulphate (99.5%), anhydrous sodium sulphate (99.0%) and silica gel 60 GF_{254} for thin layer chromatography were supplied by Merck (Darmstadt, Germany). Potassium phosphate (98%), ammonium acetate (98%), Tris–HCl, Triton X-100, sodium hydroxide, sodium chloride (99.5%), and the components of the MGB-11 were provided by Sigma–Aldrich (St. Louis, MO, USA). Other reagents (pyridine, diethyl ether, acetone, HCl, KCl, Na_2HPO_4, NaH_2PO_4, analysis grade) were supplied by Teknokroma (Barcelona, Spain). Tetrabutylammonium acetate and ammonium acetate were supplied by Fluka (Zwijndrecht, The Netherlands). Bakerbond SPE C18 columns were supplied by J. T. Baker (Deventer, The Netherlands). *N,N*-dimethylformamide (DMF) PAR grade and LC/MS grade solvents and water were supplied by Panreac (Barcelona, Spain), while acetone HPLC grade was supplied by Merck (Darmstadt, Germany). Deionized water was obtained from a Milli-Q 50 system (Millipore Corp., Milford, MA, USA). For MS calibrations a tuning mix (ESI-L low concentration tuning mix, Agilent Technologies, Santa Clara, CA, USA) was used for ESI interface and for APCI a homemade stock solution of polyethylene glycol 200, 400, 600, 1000 mix from Sigma-Aldrich (St. Louis, MO, USA) in MeOH:water (1:1).

2.3. Chlorophyll extraction

The raw material was mixed with liquid nitrogen and grinded into powder in a mortar. Then, this powder was sequentially passed through 576 meshes/cm^2 sieve, obtaining seaweed powder with particle size equivalent to 0.5 mm (Φ). Immediately, moisture content was determined by standard moisture analyser (OHAUS, MB35). For direct solvent extraction, 0.2 g of seaweed powder were mixed with 30 mL of extraction solvent, DMF:water (9:1), vortex mixed (10 s), centrifuged (6000 rpm, 3 min) and filtrated (nylon, 0.45 μm). Subsequently, one third of the final volume was mixed with the same volume of NaCl water solution (10% w/v) in a separation funnel, and the chlorophyll pigments were extracted with diethyl ether. The organic layer was isolated and concentrated to dryness in a rotary evaporator. The residue was dissolved in acetone. Samples were stored at −20 °C until HPLC-MS analysis.

2.4. Description of chlorophyll standards and isolation of chlorophyll derivatives

Chlorophyll *a* and *b* were purchased from Wako (Neuss, Germany) and Sigma-Aldrich (Madrid, Spain), respectively. Chlorophyll *d* was obtained from *Acaryochloris marina* NBRC 102967 (former mbic11017). The culture sample was extracted three times by sonicating with acetone in an ice bath for 30 min, separated and concentrated as described earlier (Kashiyama et al., 2008). Purified chlorophyll c_1 was a personal gift from Prof. Garrido (Instituto de Investigaciones Marinas, CSIC) and chlorophyll c_2 was purchased from DHI (Denmark). Chlorophyllide *a* was obtained by enzymatic de-esterification of the corresponding parent chlorophyll *a*, using partially purified chlorophyllase from *Ailanthus altissima* (Mill.) leaves. The reaction mixture contained 100 mM Tris-HCl (pH 8.5) with 0.24% (w/v) Triton X-100, chlorophyll dissolved in acetone and crude enzymatic extract in a 5:1:5 ratio (Roca, León, & de la Rosa, 2011). Mg-free derivatives (pheophytin *a* and *d*, and pheophorbide *a*) were obtained from their corresponding parent chlorophylls (chlorophyll *a* and *d*, and chlorophyllide *a*) dissolved in diethyl ether by acidification with two or three drops of 5 M HCl (Sievers & Hynninen, 1977). Oxidation with selenium dioxide of chlorophyll c_2, *b* and *a*, chlorophyllide *a*, pheophytin *a* and pheophorbide *a* in heated pyridine solution under argon produced the corresponding 13^2-hydroxy-derivatives (70 °C for 3 h) and 15^1-hydroxy-lactone-derivatives (70 °C for 7 h) (Chen, Rios, Pérez-Gálvez, & Roca, 2015; Chen, Rios, Roca, & Pérez-Gálvez, 2015). To produce pyro-pheophorbide *a* and pyro-pheophytin *a*, parent pheophorbide *a* or pheophytin *a* dissolved in pyridine solution blanketed with N_2 were directly heated at 80 °C for 4 h (Chen, Ríos, & Pérez-Gálvez et al., 2015, Chen, Rios, & Roca et al., 2015). Purpurin-18 *a* and phytyl-purpurin-18 *a* were obtained by alkaline oxidation of pheophorbide *a* and pheophytin *a* with 30% KOH in methanol (w/v) under atmospheric oxygen (Hynninen, 1973).

Epimers at C13 of chlorophyll derivatives (chlorophyll c_1', 15^1-hydroxy-lactone-pheophorbide *a*′, 13^2-hydroxy-pheophorbide *a*′, pheophorbide a′, 13^2-hydroxy-chlorophyll *b*′, chlorophyll *b*′, chlorophyll *a*′, 13^2-hydroxy-pheophytin *a*′, and pheophytin *a*′) were prepared by treatment with chloroform for two hours in a refrigerator (Watanabe et al., 1984) from the corresponding parent compounds.

2.5. Pigment identification by HPLC-API-hrTOF-MS/MS

The liquid chromatograph was Dionex Ultimate 3000RS U-HPLC (Thermo Fisher Scientific, Waltham, MA, USA). Chromatographic separation was performed as described by Chen, Ríos, and Pérez-Gálvez et al. (2015), Chen, Ríos, and Roca et al. (2015). The injection volume was 30 μL and the flow rate was 1 mL/min. A stainless steel column (200 × 4.6 mm, 3 μm particle size) packed with Mediterranea Sea18 (Teknokroma, Barcelona, Spain) was used. A split post-column of 0.4 mL/min was introduced directly on the mass spectrometer ion source. Mass spectrometry was performed using