Journal of Functional Foods 40 (2018) 400–407

Contents lists available at ScienceDirect

Journal of Functional Foods

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

In vitro digestion of chlorophyll pigments from edible seaweeds

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ARTICLE INFO

Keywords:
Bioavailability
Chlorophyll
In vitro digestion
Kombu
Nori
Sea lettuce
Seaweeds

ABSTRACT

The three most common edible seaweeds, Nori (*Porphyra umbilicalis*), Sea Lettuce (*Ulva* sp.) and Kombu (*Laminaria ochroleuca*), were subjected for the first time to an *in vitro* digestion process in order to study the digestive stability and recovery of chlorophyll pigments of the *a*, *b* and *c* series. Due to the complex and diverse chlorophyll profile in these seaweeds, new principles regarding the behaviour of chlorophylls during the *in vitro* digestion were obtained. Thus, the *in vitro* digestion conditions favoured the pheophytinisation reaction of the *a* series in comparison with the *b* and *c* series; oxidation reactions were promoted for chlorophylls instead of pheophorbides and pheophytins; the conversion of pheophytin to pheophorbide was observed for the first time during *in vitro* digestion and only occurred when the initial chlorophyll profile contained a large proportion of pheophytins. The extracellular matrix of the seaweeds was the main determining factor in the recovery of pigments after *in vitro* digestion, with significant differences between seaweed species. The novel chlorophyll behaviour observed for digestive, stability and recovery patterns obtained in the present work are applicable to other food matrices.

1. Introduction

Chlorophyll pigments, which allow plant species to convert light into biological energy, are the most abundant pigments on earth and exhibit a variety of biological actions (Roca, Chen, & Pérez-Gálvez, 2016), such as an antimutagenic effect (Simonich et al., 2007), anti-genotoxic properties (Negishi, Rai, & Hayatsu, 1997), and a potent antioxidant capacity to scavenge free radicals, preventing lipid oxidation (Lanfer-Marquez, Barros, & Sinnecker, 2005). Additionally, natural chlorophyll pigments deriving from marine algae exhibit other potential health benefits, such as neuroprotective (Ina, Hayashi, Nozaki, & Kamei, 2007) and anti-inflammatory features (Henderson & Kincaid, 1997). Despite the fact that chlorophyll is abundant in our diet, the available information about the digestive process of chlorophyll pigments is limited. This may be due to the fact that chlorophyll pigments are very sensitive to the environmental changes and prone to chemical and enzymatic changes (Schwartz & Lorenzo, 2006). Consequently, there was an assumption in the past that chlorophyll pigments could not be absorbed by the human body. However, since Egner et al. (2000) described copper chlorin e4 and chlorin e4 ethyl ester in human serum, research began on the bioavailability of chlorophyll pigments. The assumed mechanism of chlorophyll absorption proposed by Ferruzzi and Blakeslee (2007) follows similar routes to those observed for other xenobiotic compounds that require (a) efficient release of the chlorophyll from the food matrix, (b) stability for gastric and small intestinal digestive conditions, (c) solubilisation of lipophilic derivatives (micellization), (d) uptake by small intestinal absorptive epithelial cells, and (e) secretion into circulation (basal transport).

Consequently, the first step for the large-scale research of nutrient absorption (Garrett, Failla, & Sarama, 1999) is to establish the efficiency of bioaccessibility, defined as the amount of the ingested compound that is transferred during digestion from the food matrix to the micelles, measured through *in vitro* digestion protocols. Information on the bioaccessibility of chlorophyll derivatives is scarce and limited to higher food matrix plants, such as spinach leaves (Ferruzzi, Failla, & Schwartz, 2001) or pea purée (Gallardo-Guerrero, Gandul-Rojas, & Mínguez-Mosquera, 2008), where the chlorophyll profile is narrow: mainly chlorophyll *a* and *b* in the initial material with only tiny amounts of pheophytin *a* and pheophytin *b*. Finally, a study has been performed with several chlorophyll standards (Gandul-Rojas, Gallardo-Guerrero, & Mnguez-Mosquera, 2009) including chlorophyll *a (b)*, pheophytin *a (b)*, pyropheophytin *a*, pheophorbide *a* and pyropheophorbide *a*. It has been shown that de-esterification of phytol makes chlorophyll pigments more bioaccessible. This suggests the need to extend the research of the digestion process of chlorophyll pigments to a wider range of chlorophyll derivatives in a food matrix.

Marine algae or seaweeds, which have been traditionally consumed in Asian diets since ancient times, have gained popularity in European

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https://doi.org/10.1016/j.jff.2017.11.030
Received 17 March 2017; Received in revised form 19 October 2017; Accepted 21 November 2017

countries (Taboada, Millán, & Míguez, 2010). Currently, it is widely accepted that seaweeds are rich in vitamins, minerals, dietary fibre and other functional nutrients that can provide a necessary supplement to the modern diet, which mainly consists of refined food ingredients (Ferraces-Casais, Lage-Yusty, Rodríguez-Bernaldo de Quirós, & López-Hernández, 2012). Since the discovery of the many beneficial compounds present in algae, much effort has been expended in examining their nutritional aspects, including bioaccessibility of micronutrients such as iodine and carotenoid (Granado-Lorencio et al., 2009), and the overall nutritional effects on tested animals (Taboada et al., 2010). However, no reports were found associated with the digestive behaviour of chlorophyll pigments from seaweeds, even though it has already been discovered that content of marine algae is extremely high in these pigments and indeed the profile is very extensive (Chen, Ríos, Pérez-Gálvez, & Roca, 2017; Pangestuti & Kim, 2011).

In this study, the three most consumed seaweeds were selected for an *in vitro* digestion process, including Nori, Sea Lettuce and Kombu. These three species are representative of the three different classes of algae including the red, green and brown kinds, which are Rhodophytes, Chlorophytes and Phaeophytes, respectively. The aim of the study was to analyse the influence of different food matrices on the bioaccessibility of chlorophylls, as the different classes of algae show very different extracellular matrix features (Synytsya, Čopíková, Kim, & Yong, 2015). In addition, these materials provide unique, complex and complete chlorophyll profiles that allow a systematic approach to digestive properties. Nori contains only *a* series chlorophyll, Sea Lettuce contains *a* and *b* series, and Kombu, *a* and *c* series (Chen et al., 2017; Ferraces-Casais et al., 2012). Indeed, seaweeds contain a large amount of dephytylated chlorophyll such as pheophorbide (Ferraces-Casais et al., 2012), magnesium-free chlorophyll such as pheophytin and its oxidised derivatives (Koseki, Muranaka, Sakai, & Nakajima, 2002) as well as chlorophyll *a* and *b*. This allows a comparison to be made between different structures of chlorophyll related to their digestive behaviour and the effect of food matrices from different seaweed species during the digestive metabolism.

2. Materials and methods

All the following procedures were carried out under green light to avoid the photooxidation of chlorophyll pigments.

2.1. Raw material

Sea Lettuce (*Ulva* sp.) was provided by Suralgae (Cádiz, Spain) while Nori (*Porphyra umbilicales*) and Kombu (*Laminaria ochroleuca*) were provided by Algamar (Pontevedra, Spain). The three 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.

2.2. Chemicals and reagents

Sunflower oil was purchased in a local supermarket. α-Amylase (porcine pancreas, VI-B), pepsin (porcine), bile extract (porcine), lipase pancreatic (porcine), sodium chloride, sodium bicarbonate, tetrabutylammonium acetate and ammonium acetate (98%) were supplied by Sigma-Aldrich Chemical Co. (Madrid, Spain). Pancreatin (porcine) was provided by Fluka (Zwijndrecht, The Netherlands). Other reagents (acetone, potassium chloride, calcium chloride, analysis grade) were supplied by Teknokroma (Barcelona, Spain). *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. The deionized water used was obtained from a Milli-Q 50 system (Millipore Corp., Milford, MA, USA).

2.3. Sample preparation

To warranty the consistency of each sample for the independent *in vitro* digestion, samples of seaweeds were prepared according to the following procedure. Amounts of fresh dried seaweeds (15 g for Nori and Kombu, 7.5 g for Sea Lettuce) were weighed and combined with saline solution (140 mM NaCl, 5 mM KCl and 6 mM $CaCl_2$) in a ratio of 1:20 (w/v). The mixture was homogenized to obtain the consistent puree. Aliquots corresponding to ca. 0.1 g of fresh dried seaweeds for Sea Lettuce and ca. 0.2 g for Nori and Kombu were blanketed with nitrogen and stored at −20 °C before used.

2.4. In vitro digestion

The method was adopted from Garrett et al. (1999) and Gallardo-Guerrero et al. (2008) with some modifications. Samples were subjected to the oral, gastric and intestinal phase of digestion to reproduce the physiological process. Initially, 80 μL of sunflower oil free of chlorophyll pigments was added into the defrosted sample to achieve the requirement of food matrix in daily intake (around 4% of the fresh wet weight of seaweeds). The three digestive mixtures were incubated in a water bath (37 °C) where the samples were horizontally shaken at constant rate (85 rpm) for the oral (10 min), gastric (1 h) and intestinal (2 h) phase. From the first phase, the simulated saliva was obtained by dissolving α-amylase (2041 U/g fresh wet weight of raw material) in saline solution (140 mM NaCl, 5 mM KCl, pH 7.0). The gastric phase was initiated by adding saline solution to reach a final concentration of 2.4 mg/mL pepsin and adjusting the pH to 2.0 by adding some drops of 0.1 M HCl. Subsequently, the pH was adjusted to 6.0 by adding $NaHCO_3$ (0.9 M) to initiate the intestinal phase including bile salts (2.4 mg/mL), pancreatin (0.4 mg/mL) and lipase (0.2 mg/mL) dissolved in $NaHCO_3$ (0.1 M) solution. The final volume of the digesta was around 35 mL. Reactions were stopped by placing tubes in ice bath and aliquots (2 × 5 mL) of digesta were collected. All the samples were blanketed with nitrogen and placed at −20 °C until analysis.

2.5. Pigment extraction

The raw fresh dried seaweeds were grinded with liquid nitrogen and passed through 576 meshes/cm^2 sieves (particle size equivalent to 0.5 mm (Φ)), and the moisture content was measured. 0.2 grams of seaweed powder were extracted with 30 mL of DMF:water (9:1), vortex mixed (10 s), centrifuged (6000 rpm, 3 min), filtrated (nylon, 0.22 μm) and stored at −20 °C until HPLC analysis (Chen et al., 2017).

The digesta samples were lyophilized (Virtis, Benchtop K) before pigment extraction. Then, 200 μL distilled water was added to soften the porous residue and the mixture was shaken for 5 min. Additional 200 μL of DMF was added to the mixture and shacked again 5 min. Next, 1.6 mL acetone was added and the mixture immersed in an ultrasonic bath (10 min, 720 W). The solvent layer after filtration was directly analysed by HPLC.

2.6. Pigment identification and quantification by HPLC-UV–Visible

The pigments were separated by reversed-phase HPLC using a Hewlett-Packard HP 1100 liquid chromatograph. A Mediterranea Sea18 column (200 × 4.6 mm, 3 μm particle size) was used (Teknokroma, Barcelona, Spain) protected by a guard column (10 × 4.6 mm) packed with the same material. Separation was performed using the elution gradient described by Roca, Gandul-Rojas, and Mínguez-Mosquera (2007) with the mobiles phases: A, water/ion pair reagent/methanol (1/1/8, v/v/v) and B, methanol/acetone (1/1, v/v). The ion pair reagent was 0.05 M tetrabutylammonium and 1 M ammonium acetate in water. The on-line UV–visible spectra were recorded from 350 to 800 nm with the photodiode–array detector and sequential detection was performed at 410, 430, 450 and 666 nm. Data were collected and