Journal of Functional Foods 41 (2018) 25–33

Contents lists available at ScienceDirect

Journal of Functional Foods

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

In vitro bioavailability of chlorophyll pigments from edible seaweeds

Kewei Chen, María Roca*

Food Phytochemistry Department, Instituto de la Grasa, Consejo Superior de Investigaciones Científicas (CSIC), University Campus, Building 46, Carretera de Utrera km. 1, Sevilla 41013, Spain

ARTICLE INFO

Keywords:
Seaweed
Chlorophyll
Bioavailability
Micellarisation
Absorption
Caco-2 cells

ABSTRACT

For the first time, the uptake of chlorophyll pigments from the main edible seaweeds (Nori, Sea Lettuce and Wakame) has been investigated. During the micellarisation process, dephytylated chlorophylls were favoured over phytylated chlorophylls (*a* and *c* series were favoured over *b* series and oxidised chlorophylls were preferentially micellarised). This is the first time chlorophyll *b* derivatives have been found to be resistant to the *in vitro* digestion of the food matrix, indicating they are also potentially absorbable by enterocytes during the ingestion of green vegetables and fruits. Nori chlorophylls stand out as the most bioaccessible, followed by those in Sea Lettuce and Kombu. During the Caco-2 cell absorption process, dephytylated chlorophyll derivatives were also favoured over phytylated ones, with pheophorbide *c* being the most absorbable chlorophyll pigment. It is also the first time that chlorophyll oxidation reactions have been observed during cell absorption. The uptake of chlorophyll derivatives from edible seaweeds resulted in Caco-2 cell lines with a chlorophyll profile dominated by dephytylated and oxidised derivatives.

1. Introduction

Chlorophyll pigments, the most abundant natural pigments in nature with over 10^9 tonnes estimated to be biosynthesised and degraded every year on earth (Hendry, Houghton, & Brown, 1987), are consumed in the human diet when ingested along with green vegetables and fruits. According to a nine-year follow-up investigation (Balder et al., 2006), it is estimated that 26–86 mg of chlorophyll pigments are assimilated by the human body each day. In addition, chlorophyll pigments show many health benefits (Ferruzzi & Blakeslee, 2007), such as good ability to chelate with some chemical carcinogens and mutagens (Breinholt, Schimerlik, Dashwood, & Bailey, 1995; Dashwood, Yamane, & Larsen, 1996; Egner et al., 2001; Jubert et al., 2009; Simonich et al., 2007; Tachino et al., 1994), reducing cancer risk, and potent antioxidant activity (Cervantes-Paz et al., 2014; Hsu, Chao, Hu, & Yang, 2013; Lanfer-Marquez, Barros, & Sinnecker, 2005), scavenging free radicals. Interestingly, chlorophyll derivatives incorporated in micelles are employed for intestinal imaging purposes (Zhang et al., 2016)

Historically, it has been assumed that chlorophylls are not assimilated by animals, as they are found in faeces after “green” diets rich in chlorophyll derivatives (Ashby et al., 2003; Barnes, Rasmussen, Petrich, & Rasmussen, 2012; Dashwood & Guo, 1995; Lee et al., 2010). For the first time, Egner et al. (2000) found copper chlorin e_4 and copper chlorin e_4 ethyl ester in the serum samples of a clinical trial of a copper chlorophyllin (mixture of modified water-soluble chlorophyll derivatives) study, indicating directly that chlorophyll derivatives can be absorbed by human bodies. In spite of this, there have been few investigations into *in vivo* chlorophyll assimilation in the gastro-intestinal system. The available data is limited to the co-ingestion of chlorophyll compounds with potential carcinogens like haem (De Vogel, Jonker-Termont, Katan, & Van der Meer, 2005), aflatoxin and others (Blum et al., 2003; Simonich et al., 2007) in rat models where the main focus was on mechanisms to suppress the cancer occurrence. Therefore, it is necessary to carry out research into the uptake of chlorophyll pigments. In this aspect, data are limited to research on certain chlorophyll derivatives from spinach leaves (Ferruzzi, Failla, & Schwartz, 2001), peas (Gallardo-Guerrero, Gandul-Rojas, & Mínguez-Mosquera, 2008) and pure chlorophyll standards (Gandul-Rojas, Gallardo-Guerrero, & Mínguez-Mosquera, 2009), indicating the importance of molecular structure in their uptake. Like other lipophilic micronutrients, chlorophyll availability requires incorporation into micelles to form the aqueous phase for effective intestinal absorption. As for carotenoids, similar to chlorophyll in some physiochemical properties, it has been shown that the solubilisation of carotenoids in mixed micelles is mostly dependent on the physiochemical properties of the carotenoid structure, food matrix, micelle formation from bile salts and lipid hydrolysis (Garrett, Failla, & Sarama, 1999; Sy et al., 2012; Tyssandier, Lyan, & Borel, 2001). However, there has been little study into this area in relation to the micellarisation process of chlorophyll pigments. Only a few

* Corresponding author.
E-mail addresses: covichen@hotmail.com (K. Chen), mroca@ig.csic.es (M. Roca).

https://doi.org/10.1016/j.jff.2017.12.029
Received 6 October 2017; Received in revised form 12 December 2017; Accepted 12 December 2017

papers (Ferruzzi et al., 2001; Gallardo-Guerrero et al., 2008) have analysed the micellarisation of specific chlorophyll derivatives in food matrices (spinach and peas).

Caco-2 cells, a continuous cell line of heterogeneous human epithelial colorectal adenocarcinoma cells (Fogh & Trempe, 1975), are typical research tools for nutrient absorption. Ferruzzi, Failla, and Schwartz (2002) demonstrated the accumulation of copper chlorophyllin by Caco-2 human intestinal cells and its basolateral efflux by differentiated cells grown on inserts. In relation to natural chlorophyll pigments, micellar pheophytins from spinach puree (Ferruzzi et al., 2001) and peas (Gallardo-Guerrero et al., 2008) were also found to be absorbable by Caco-2 cells. Chlorophyll derivatives tested in both food matrices for cell absorption were mainly pheophytins, while the green food ingested daily contains a rich profile of chlorophyll derivatives originated during chlorophyll metabolism, processing or storage (Chen, Ríos, Pérez-Gálvez, & Roca, 2017; Ferruzzi & Blakeslee, 2007; Kohata, Hanada, Yamauchi, & Horie, 2014; Van Breemen, Canjura, & Schwartz, 1991). Until now, the food reference material used to research the intestinal absorption of chlorophyll is the typical green plant (peas and spinach), which essentially means chlorophyll *a* and *b*. Consequently, this suggests the need to extend the investigation of cell absorption of chlorophyll pigments to include a wide range of chlorophyll derivative distribution. The edible seaweeds Nori (*Porphyra umbilicales*), Kombu (*Laminaria ochroleuca*) and Sea Lettuce (*Ulva* sp.) have been shown to be an excellent reference material to analyse chlorophyll stability during *in vitro* digestion (Chen & Roca, 2018) due to their rich chlorophyll profile (Chen et al., 2017). In addition, it has been established elsewhere that the food matrix is a determining factor in the bioaccessibility of micronutrients (Holst & Williamson, 2008; Lemmens et al., 2014) or the cell absorption process (Gallardo-Guerrero et al., 2008); consequently, edible seaweeds represent a novel research material due to their rich content in edible fibres (water-soluble or not) (Davis, Volesky, & Mucci, 2003; Kumar & Singh, 1979) that are released in the digestion environment to impact the micellarisation and cell absorption process of chlorophyll pigments.

In order to analyse the uptake of a diverse chlorophyll food matrix, chlorophyll pigments obtained from an *in vitro* digestion of edible seaweeds were subjected to the micellarisation process, followed by Caco-2 cell analyses to evaluate their properties to solubilise in mixed micelles and to be absorbed by human intestinal cell lines. Comparisons were made between different types of chlorophyll derivatives and between different kinds of edible seaweeds to learn more about the uptake of the chlorophyll pigments consumed daily.

2. Materials and methods

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

2.1. Raw materials

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 macroalgae 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

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 deionised water used was obtained from a Milli-Q 50 system (Millipore Corp., Milford, MA, USA). Sodium chloride, α-amylase (porcine pancreas, VI-B), pepsin (porcine), bile extract (porcine), lipase pancreatic (porcine), tetrabutylammonium acetate, ammonium acetate (98%), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), butylated hydroxytoluene (BHT), sodium taurocholate, 2-Hexyl-1-cyclopentanone thiosemicarbazone (BLT1), trypsin (500 BAEE units porcine trypsin), fetal bovine serum, penicillin, streptomycin, L-glutamine, nonessential amino acids, Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and phosphate-buffered saline (PBS) were provided by Sigma-Aldrich Chemical Co. (Madrid, Spain). Pheophorbide *a* and chlorophyll *a* were purchased from Wako Chemicals (Tokyo, Japan). Other reagents (acetone, potassium chloride, analysis grade) were supplied by Teknokroma (Barcelona, Spain).

2.3. In vitro digestion

Three edible seaweeds including Nori, Sea Lettuce and Kombu were subjected to the *in vitro* digestion and the procedure is detailed in Chen and Roca (2018). The protocol briefly consists on simulated oral, gastric and intestinal phase of the digestion process to reproduce the physiological conditions. For the first step, α-amylase (2041 U/g) in saline solution (140 mM NaCl, 5 mM KCl, pH 7.0) was incubated during 10 min at 37 °C. After oral phase, the gastric phase was performed at pH 2.0 with a final concentration of 2.4 mg/mL pepsin during 1 h at 37 °C. Finally, the pH was readjusted to 6.0 to initiate the intestinal phase that required the incubation during 2 h with 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. Aliquots (2 × 5 mL) of digesta were collected, blanketed with nitrogen and placed in −20 °C until analysis.

2.4. Obtaining aqueous micellar fraction (AMF)

Following the micellarisation at the intestinal phase of the *in vitro* digestion, the aqueous micellar fraction (AMF) was separated by ultracentrifugation. After aliquots of digesta (2 × 5 mL) were collected for chlorophyll analysis, AMF was obtained by centrifugation of the rest of the digesta at 50,000*g* at 4 °C for 90 min (Beckman model L7-65 Ultracentrifuge) to remove the solid compound and the upper oil droplets. AMF was collected carefully using a Pasteur pipette and filtrated (0.2 μm) to remove other interfering aggregates. Aliquots (2 × 5 mL) of AMF were used for the chlorophyll analysis. All the samples were blanketed with nitrogen and placed below −20 °C until analysis.

2.5. Culture of Caco-2 cells

Caco-2 (Caucasian colon adenocarcinoma) cells were purchased from Sigma-Aldrich Chemical Co. (Madrid, Spain). Stocks were maintained in complete medium described by comprising high glucose DMEM (pH 7.4), containing 4.5 g/L glucose and supplemented with penicillin (100 units/mL), streptomycin (100 μg/mL), L-glutamine (0.292 mg/mL), nonessential amino acids (10 mL/L of a 100 × stock-solution), HEPES buffer (10 mmol/L), and 10% (v/v) heat-inactivated fetal bovine serum. Cells were sub cultured at 70%-80% confluence with trypsin and seeded, in 75 cm^2 flasks with Nunclon-treated surface (Nunc A/S), at densities of 3×10^4 cells/cm^2, and incubated at 37 °C in a humidified atmosphere of air/carbon dioxide (95:5, v/v). All the experiments used highly differentiated monolayers at passages 44–54, 11–14 days after reaching confluency. Medium was replaced every 2–3 days. Prior to the chlorophyll absorption experiments, the last medium changes were carried out using serum-free medium.

2.6. Uptake of micellar chlorophyll derivatives by Caco-2 monolayers

Assays for absorption of micellar chlorophyll derivatives by Caco-2 cell monolayers were performed with the analytical conditions described by Garrett et al. (1999) and Gallardo-Guerrero et al. (2008). Prior to examining the uptake of micellar chlorophyll derived from the