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Iodine speciation in iodine-enriched microalgae *Chlorella vulgaris**

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Abstract: The characterization of iodine species in the microalgae *Chlorella vulgaris* after cultivation with different potassium iodide concentrations was performed using the coupling of multidimensional chromatography (size exclusion chromatography, SEC, and anion exchange chromatography, AEC) with inductively coupled plasma-mass spectrometry (ICP-MS) detection. Two iodine fractions, water-soluble and macromolecular fractions, were obtained using a sequential extraction scheme based on chemical reagents. Most iodine species separated from the water-soluble fraction with SEC-ICP-MS (mass range from 0.5 to 100 kDa) are present in inorganic forms (peak III), although the other two peaks were detected (peaks I and II). The application of AEC to the isolated peak III fraction allows the characterization of IO_3^- (about 25 %) and I^- (about 75 %). The application of SEC-ICP-MS (mass range from 10 to 1200 kDa) to the macromolecular fraction reveals the presence of four peaks from the void volume to about 67 kDa, a peak is located about 600 kDa. The mass balance of iodine in the different fractions obtained from the microalgae raw stuff shows that the water-soluble fraction represents 66.7 % of total iodine in microalgae, but the macromolecular fraction only contains 13.3 %, both summing up for 79.9 % of the total amount of iodine, which confirms the suitability of the separation scheme. Further studies have to be focused on the purification of the isolated fractions and their identification by tandem MS.

Keywords: Chlorella; functional foods; iodine speciation; metallomics; microalgae.

INTRODUCTION

Iodine is a critical element for the thyroid hormones behavior, whose presence is necessary to monitor biological and environmental samples. Iodine deficiency in human food or animal feed may lead to organism malfunction, such as rate enhancement, growth and development, carbohydrate metabolism, oxygen consumption, protein synthesis, fetal neurological development, and so on, in which functions iodine is complemented by selenium [1].

In the iodine cycle, several enzymes promote the incorporation of monoiodothyrosine (MIT) and diiodothyrosine (DIT) at the moieties of the protein thyroglobulin, where, depending on their relative position, MIT and DIT produce thyroxine (T_4), triiodothyronine (T_3), and the biologically inactive reverse T_3 (r-T_3), which after proteolysis are leached and triggered into the blood stream. In the plasma the thyroid hormones are transported by proteins and participate in an activation–deactivation mecha-

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nism [2] in which deiodinase, a Se-containing enzyme, transforms the prohormone T_4 into the active hormone T_3 by removing an iodine atom onto the outer ring. Inactivation of thyroid hormones occurs by removal of an iodine atom onto the inner ring of T_4, which converts T_4 in the r-T_3, or, alternatively, the active T_3 into the inactive diiodothyronine (T_2).

Iodine concentration in food needs to be controlled, because daily intake of this element recommended for adults ranging from 150 to 200 μg is seldom achieved [3], and iodine deficiency is the primary cause of goiter and can lead to cretinism [4]. The main source of iodine in nutrition is sea fish, milk, and meat, however, the element is added to the feed of cows to enhance milk production, which explains the high levels of this element in milk [5]. In East Asian countries, mainly Japan and China, algae (e.g., *Laminaria*) are used for consumption which can produce iodine overdose and, as a consequence, a high prevalence of goiter and hypothyroidism [6]. The opposite situation generally occurs in inland areas with iodine deficiency, which has resulted in iodine incorporation in table salt (15–25 μg g^{-1}) or different dietetic foodstuff [7].

Iodine bioavailability depends on the chemical forms of the element. The content of total iodine, water-soluble and organic non-water-leachable iodine in several macroalgae has been determined [8]. In *Sargassum*, only 40 % of iodine is water-leachable, mainly as inorganic iodide and iodate. The content and distribution of iodine in several biological macromolecules was established. The element is mainly bound to proteins and in minor percentage to polyphenols, pigments, and polysaccharides (algin and fucoidan). Chai et al. [8] have proposed a chemical leaching scheme to isolate iodine-linking proteins, because there are thousands of proteins in seaweed. Acetone was used to leach pigments and then $CaCl_2$ and caffeine to fix the polysaccharide algin and polyphenols, respectively. Subsequently, treatment with Tris-HCl buffer, SDS (sodium dodecyl sulfate), and NaN_3 was used to break cell walls and protect leachate against bacteria. Finally, proteins were precipitated with $(NH_4)_2SO_4$, redissolved with water and dialyzed, the resulting extract was lyophilized.

The microalgae *Chlorella vulgaris* has been studied by Burianova et al. [3] for iodine accumulation and latter use as food supplement. There are thousands of microalgae that can be used for this purpose, but *Chlorella* grows very fast and it is easy to monitor the iodine intake process. A chemical scheme has been proposed to separate water-soluble iodine species (iodide and iodate) from organic iodine, protein and volatile iodine compounds. Microalgae can accumulate until 5000 mg of I kg^{-1}, mainly as water-soluble iodine (about 90 %) in the form of iodide.

In the present work, a preliminary study for the characterization of iodine species accumulated by *C. vulgaris* has been performed. The use of multidimensional chromatography (size exclusion chromatography, SEC, and anion exchange chromatography, AEC) with inductively coupled plasma-mass spectrometry (ICP-MS) detection has been a powerful tool for this purpose. In addition, the optimization of iodine accumulation in *Chlorella* at laboratory scale was considered. The final aim is to get suitable information for the preparation of iodine functional foods based on this microalga.

EXPERIMENTAL

Standard solutions and reagents

Analytical-grade reagents purchased from Sigma-Aldrich (St. Quentin Fallavier, France) were used throughout unless specified otherwise. 18 MΩ Milli-Q water (Millipore, Bedford, MA, USA) was used throughout. The Tris-HCl buffer was prepared by dissolving 30 mM of Tris [tris(hydroxymethyl)-aminomethane] in water and adjusting the pH to 7.0 by the addition of hydrochloric acid (1:10, v/v). The buffer solution was degassed in an ultrasonic bath before use.

Instrumentation

Chromatographic separations were carried out using an HP Model 1100 HPLC pump (Hewlett-Packard, Wilmington, DE, USA) as the sample delivery system. Injections were performed using a Model 7725 injection valve with a 50-µl injection loop (Rheodyne, Cotati, CA, USA). All the connections were made of polyether ether ketone (PEEK) tubing (id 0.17 mm). Analyte species were separated on 10 × 300 mm × 13 µm Superdex-75 and Superdex-200 columns (Pharmacia Biotech, Uppsala, Sweden) with an exclusion limit of 100 kDa (an effective separation range of 0.5 and 80 kDa) and an exclusion limit of 1300 kDa (an effective separation range of 10 and 600 kDa), respectively [9]. The columns were calibrated (UV detection was used) with the following standards: Gly6 (360 Da), gastrin rat I (2126 Da), rabbit liver metallothionein I (7000 Da), ribonuclease A (13 700 Da), chymotrypsinogen A (25 000 Da), bovine albumin (Mr 66 000), and thyroglobulin (Mr 660 000).

Elemental detection was performed using an Agilent 7500c ICP-MS (Agilent, Tokyo, Japan). The HPLC-ICP-MS on-line coupling was performed by connecting the outlet of the chromatograph to the nebulizer inlet of the ICP-MS instrument. A model 5804 R centrifuge (Eppendorf, Hamburg, Germany) was used to accelerate the phase-separation process in the extraction of the compounds.

Sample preparation

Microalgae *C. vulgaris* was kindly provided by the Group of Biochemistry and Biotechnology of Photosynthetic Organisms from the University of Huelva. Standard cultures were performed in a culture room, were grown in mineral liquid medium at 25 °C, bubbled with air containing 5 % (v/v) CO_2, and continuously illuminated with white light from fluorescent lamps (22 W m^{-2}, at the surface of the flasks). The composition of the culture medium was described previously [10]. Algae were cultivated in the presence of potassium iodide. The cell pellets were lyophilized for later analysis.

Total iodine determination

The analysis of total iodine content was performed on 0.250 g of lyophilized microalgae or extracts by digestion in a microwave-accelerated reaction system, adding 10 ml of ultrapure water and 10 ml of tetramethylammonium hydroxide to a 55-ml microwave Teflon vessel. The poly(tetrafluoroethylene) (PTFE) vessels were closed and introduced into the microwave oven. The mineralization was carried out at 1000 W from room temperature ramped to 200 °C in 10 min and with holding for 5 min at this temperature [11]. Then, solutions were made up to 25 ml and analyzed by ICP-MS. The method of standard additions was applied for the quantification of the iodine content. Rh was used as the internal standard.

ICP-MS measurement conditions [nebulizer gas flow, radio frequency (RF) power, and lens voltages] were optimized daily using a standard built-in software procedure. An aqueous solution of potassium iodide 10 µg l^{-1} was used for sensitivity optimization. Typical examples of the optimum measurement conditions are a nebulizer gas flow of 1.05 l min^{-1}, ICP RF power of 1.1 kW, and a lens voltage of 9 V. The same instrumental conditions were employed when ICP-MS was used as the chromatographic detector.

Speciation of iodine by SEC and ICP-MS

A Superdex 75 column with 100 kDa exclusion limit and 0.5–80 kDa of effective separation range was used for this purpose. A Tris-HCl buffer (pH 7.0) was used as mobile phase at a flow rate of 0.75 ml min^{-1}. A sample aliquot of 50 µl was injected. The eluate from the column was fed directly into the ICP-MS nebulizer using a PEEK tube of 50 cm. The ^{127}I isotope was monitored.

Speciation of inorganic iodine by AEC and ICP-MS

An Agilent 1100 liquid chromatography module equipped with a column (Agilent, Tokyo, Japan) guard column (G3154A/102) and a separation column (G3154A/101) was used for species separation. The inner diameter of the main column was 4.6 × 150 mm and the guard column was 4.6 × 10 mm and the column temperature was set at 20 °C. A mobile phase composed of 20.0 mM NH_4NO_3 at pH 5.6 was used for the separation of iodate and iodide with a flow rate of 1.0 ml/min. The outlet of the separation column was directly connected to the nebulizer of the ICP-MS unit.

RESULTS AND DISCUSSION

Suitable conditions for the accumulation of iodine in *Chlorella* are strongly depending on iodine concentration in the culture medium. The best experimental conditions for iodine accumulation were obtained for concentrations of 80 μg ml^{-1} of the element in the culture medium as iodide (Fig. 1). For higher concentrations, microalgae grows with difficulty and finally collapses.

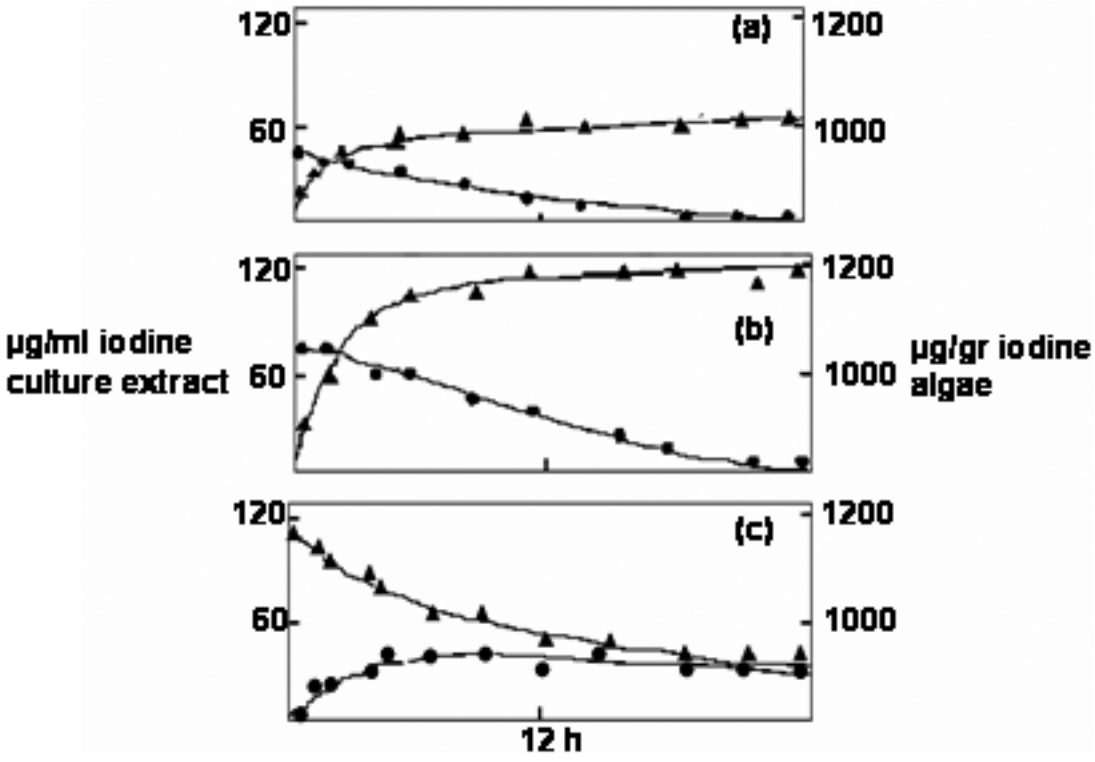

Fig. 1 Accumulation of iodine in the microalgae *C. vulgaris* under different concentration of potassium iodide in the culture. (a) 50 μg ml^{-1}; (b) 80 μg ml^{-1}; (c) 120 μg ml^{-1}.

Using optimal conditions, the iodine species present in *Chlorella* was studied using a scheme that combines the proposals from Chai [8] and Burianova [3] (Fig. 2). The use of nonaqueous solvents such as acetone or chloroform at the beginning of sequential fractionation was eliminated to avoid potential changes in the iodine molecules that make further identification of molecules by MS difficult. Therefore, water was used for leaching water-soluble molecules. The remaining treatment of residue isolated in step II intends to isolate iodine-linking macromolecules present in the algae. In the water-soluble fraction (II-a), inorganic forms of iodine (iodide from cultivation, iodate from photooxidation of iodide) and water-soluble polar organic compounds (iodizated aminoacids, peptides, and eventually water-soluble proteins) are expected. In the water-insoluble fraction (II-b), the presence of organic-bounded iodine species is assumed, especially iodine-binding proteins, which were leached with Tris-HCl buffer (pH 9) containing 0.1 % SDS, and precipitated in saturated $(NH_4)_2SO_4$ solution. Finally, an iodine-linking protein fraction was achieved by resolubilization in water and purification with dialysis (fraction V-a). Other small fractions separated along the scheme (III-b, IV-b, and V-b) have not been considered in the present study and will be considered in the future.

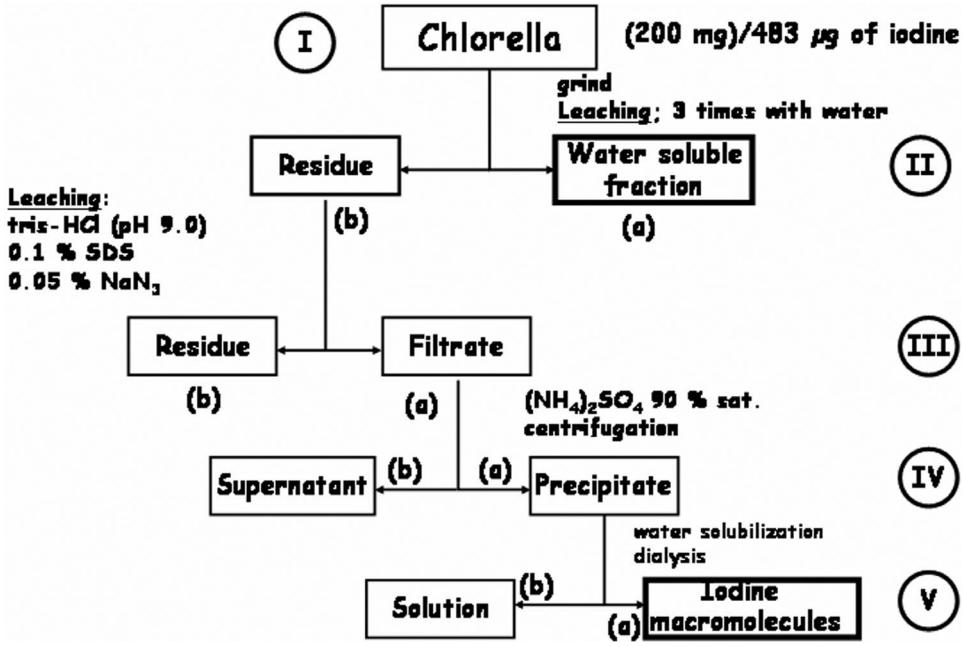

Fig. 2 Scheme for the extraction of iodine water-soluble fraction (II-a) and iodine macromolecular fraction (V-a) from *C. vulgaris.*

Molecular size distribution of aqueous fraction (fraction II-a)

A chromatographic separation based on SEC with ICP-MS detection using a Superdex 75 column with 100 kDa exclusion limit and 0.5–80 kDa of effective separation range was used for this purpose. A Tris-HCl buffer (pH 7.0) was used as mobile phase at a flow rate of 0.75 ml min^{-1}. Detection by ICP-MS allows sensitive identification of iodine-containing molecules. Three peaks were observed (Fig. 3b): peak I, in the exclusion limit, peak II eluted at mass range around 600 Da, and peak III with a retention time similar to iodide in Fig. 3a. This chromatographic system was used to collect peak III that was later submitted to a second chromatographic separation by AEC and ICP-MS detection.

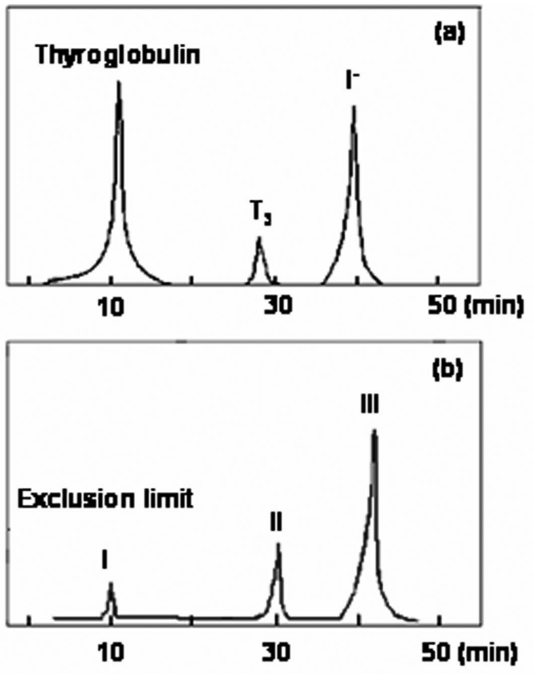

Fig. 3 SEC-ICP-MS chromatogram (Superdex 75 column) of iodine water-soluble fraction. (a) Separation of a mixture of thyroglobulin, T_3 and I^-, used as standards; (b) separation of microalgae water-soluble extract.

AEC analysis of peak III

AEC study of peak III was performed with NH_4NO_3 (pH 5.6) as mobile phase and ICP-MS as detector [AEC(HPLC)-ICP-MS]. Figure 4 shows the results obtained, only iodide and iodate were detected, with retention times matching with the corresponding standards. Iodide is the most abundant species and represents about 75 % of total iodine in the fraction, iodate accounts for 25 %. Further studies are necessary for fractions I and II of Fig. 3b. Due to the unknown character of these iodine species, identification with MS is necessary; no previous information is provided in the literature about these species, and their identification is a difficult task.

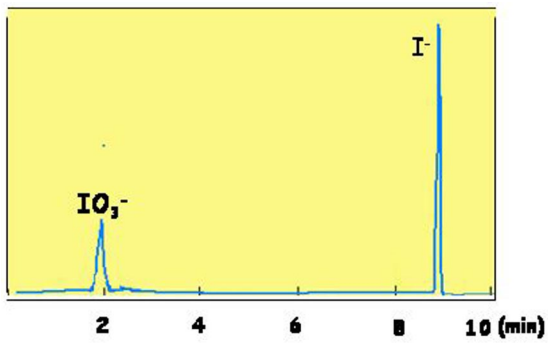

Fig. 4 AEC separation of inorganic iodine (peak III from Fig. 3).

Molecular size distribution of macromolecular fraction

The macromolecular fraction (fraction V-a, in Fig. 3) was submitted to a SEC separation using the Superdex 200 column, with an effective separation range from 10 to 600 kDa and exclusion limit at 1300 kDa. Tris-HCl buffer was used as mobile phase with pH 7.0 and a flow rate of 0.75 ml min^{-1}. The results are shown in Fig. 5. Four peaks can be observed: at the exclusion limit, at about 600, 160, and 60 kDa. However, we do not yet have information about the identity of substances eluted at these retention times, and further studies are necessary to purify these fractions with a second chromatographic orthogonal separation and identification of purified fractions with tandem MS. The metallomics approach proposed for metal-linked biomolecules related to biological functions can be used for this purpose [12–14].

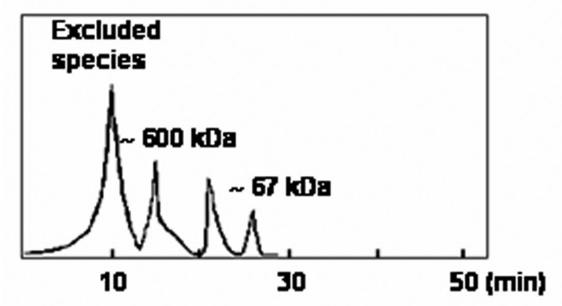

Fig. 5 SEC-ICP-MS chromatogram (Superdex 200 column) of iodine macromolecular fraction.

Mass balance of iodine in the fractions

In order to perform a quality control of the fractionation scheme performed with iodine species extracted from microalgae, a mass balance referred to total iodine amount in the *Chlorella* pellet (fraction I, Fig. 2), water-soluble fraction (fraction II-a) and iodine macromolecule fraction (fraction V-a). For these purposes, aliquots from these fractions were digested and the total amount of iodine measured by ICP-MS. The total amount of iodine, 483 μg, in microalgae raw stuff used for the study (fraction I), was distributed between fraction II-a), 320.7 μg, which represents 66.7 %, and fraction V-a which corresponds to 13.3 % of iodine, which sums for 79.9 % of the total amount of this element. About 20 % of remaining iodine was spread among fractions II-b, III-b, IV-b, and V-b (Table 1), which have not been considered in the present study but represent a considerable contribution to total iodine present in microalgae that should be considered in further studies. Total recovery of iodine in isolated fractions II-a, III-b, IV-b, V-a, and V-b reaches about 92.4 %. The two main fractions II-b and V-a, studied in more detail in the present work, account for 79.7 %. These figures confirm the suitability of the separation scheme.

Table 1 Mass balance of iodine in the different fractions.

Fraction	Subfraction	Amount of iodine (μg)	Observations	Recovery (%)
I		483		
II	II-a	320.7	Iodine water-soluble fraction	66.4
	II-b	136.3		
III	III-a	84.3		
	III-b	46.2		9.5
IV	IV-a	70.1		
	IV-b	11.1		2.2
V	V-a	64.3	Iodine macromolecules	13.3
	V-b	4.5		1.0
Total				92.4

CONCLUSIONS

The microalgae *C. vulgaris* can be used for the accumulation when cultured in iodide solution. Two main fractions can be isolated by successive leaching of microalgae stuff with water (water-soluble fraction) and SDS solution (iodine macromolecular fraction). Water-soluble fraction contains 66.4 % of iodine mainly distributed between I^- (75 %) and IO_3^- (25 %), although another small peak reveals the presence of small amounts of other iodine species in this fraction. The iodine macromolecular fraction represents about 13 % of total iodine in the microalgae stuff material and shows several peaks in the SEC-ICP-MS chromatogram, with molecular mass ranging from 60 to 600 kDa, which will be purified, isolated, and identified in further studies based on the use of tandem MS. Although a suitable amount of iodine is accumulated in the algae, additional research can allow optimal iodine enrichment in order to produce functional food suitable for human consumption. In addition, it is necessary to check the metabolism and bioavailability of iodine-based microalgae foods in humans, and establish possibilities for potential use as foodstuff.

The application of ICP-MS as detector of the chromatographic separations represents a valuable tool for characterizing the presence of iodine-linking molecules in cell-based samples as is the case of microalgae *C. vulgaris.*

ACKNOWLEDGMENTS

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