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Speciation of iodine-containing proteins in *Nori* seaweed by gel electrophoresis laser ablation ICP–MS

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ABSTRACT

An analytical approach providing an insight into speciation of iodine in water insoluble fraction of edible seaweed (*Nori*) was developed. The seaweed, harvested in the Galician coast (Northwestern Spain), contained 67.7 ± 1.3 μg g^{-1} iodine of which 25% was water soluble and could be identifies as iodide. Extraction conditions of water insoluble residue using urea, NaOH, SDS and Triton X-100 were investigated. The protein pellets obtained in optimized conditions (after precipitation of urea extracts with acetone), were digested with trypsin and protease XIV. Size exclusion chromatography-ICP–MS of both enzymatic digests demonstrated the occurrence of iodoaminoacids putatively present in proteins. Intact proteins could be separated by gel electrophoresis after an additional extraction of the protein extract with phenol. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) with laser ablation ICP–MS detection of ^{127}I indicated the presence of iodine in protein bands corresponding to molecular masses of 110 kDa, 40 kDa, 27 kDa, 20 kDa and 10 kDa. 2D IEF-SDS PAGE with laser ablation ICP–MS ^{127}I imaging allowed the detection of 5 iodine containing protein spots in the alkaline p*I* range.

1. Introduction

Iodine is an essential trace element for humans. It is a component of thyroid hormones triiodothyronine (T_3) and thyroxine (T_4) necessary for the regulation of metabolism, growth and neuronal development. The WHO (the World Health Organization) recommends a daily intake of about 150 μg of iodine for healthy adults [1]. Iodine deficiency disorders include goiter and cretinism [2]. The main source of iodine is oceans with an average concentration of 45–60 μg L^{-1}. Seafood, and particularly seaweed, can accumulate high amounts of iodine [1,3–5]. Seaweeds are considered as an interesting candidate for the development of iodine-containing functional foods and nutraceuticals [6].

In terms of speciation, iodine is usually mostly present in the water-soluble fraction (up to 99% with iodide being the major species 61–93% [7–11] accompanied by iodate (1.4–4.5%) and organic iodine compounds from 5% to 37%). Data on iodine-containing macromolecules present in seaweed are scarce. Early studies put forward a hypothesis that iodine could be metabolized by algae to iodinated amino acides (monoiodotyrosine – MIT, diiodotyrosine – DIT, diiodothyronine – T_2 and T_4) [11,12]. The presence of iodinated aminoacids was confirmed in an enzymatic extract of Wakame algae by reversed-phase HPLC ICP–MS by Shah et al. [9]. Hou et al. [12] employed a series of fractionation procedures to find out that the most abundant fraction of iodine was bound to proteins, followed by pigments and polyphenols with only a minor fraction linked to polysaccharides. Gómez-Jacinto et al. [10] showed evidence by size-exclusion chromatography with iodine specific detection (ICP–MS) for the presence of iodine in macromolecular compounds. No data on the possible differences of bioavailability of iodine mineral and macromolecular species have been reported so far.

Denaturating [13] and non-denaturating [14] gel electrophoresis protocols using the monitoring of heteroatom by LA-ICP MS have become a popular approach for the screening of seleno- and metal-containing proteins on biological samples [15]. The only application of this method for iodine-containing species concerned the detection of proteins iodinated by means of potassium triiodide [16]. The objective of this work was to optimize 1D and 2D gel-based electrophoretic protocols with laser ablation ICP–MS detection for speciation of protein-bound iodine in algae from the *Nori* family, one of most popular edible seaweeds popularly known for its use in sushi. Particular attention was given to the assessment of matching the extraction conditions with those required

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for efficient separation. Complementary data were acquired by size-exclusion ICP–MS of protein trypsin digests to confirm the exact iodine binding.

2. Experimental

2.1. Instrumentation

An Agilent 7500cs Series ICP–MS (Agilent, Tokyo, Japan) equipped with a Micromist nebulizer and a Peltier-cooled (2 °C) double pass Scott spray chamber was used for total iodine determination in raw seaweed, its extracts and protein pellets. An Agilent 1100 Series HPLC pump (Agilent, Palo Alto, CA) was coupled with the ICP–MS system for iodine speciation. The details of the chromatographic columns used are given in Table 1. The columns were supplied by GE Healthcare Life Sciences (Uppsala, Sweden). SDS PAGE was carried out on TV100 mini gels (7.5 cm × 8 cm) from Biostep (Jahnsdorf, Germany). For two-dimensional GE, isoelectric focusing (IEF) separation was done on horizontal electrophoresis system IEF–SYS and second dimension was performed on vertical electrophoresis system TV400 maxi gels (16.5 cm × 17.5 cm), both from Biostep. Immobiline™ DryStrip gels of 11 cm of a linear pH ranging from 3 to 10 (GE Healthcare Life Sciences) were used. Gels were dried in a Hoefer Slab gel dryer GD2000 from Amersham Biosciences (Uppsala, Sweden). A NewWave research (Fermont, CA) UP-213 laser ablation system was coupled with the ICP–MS instrument for iodine detection on gels obtained after either SDS PAGE or bi-dimensional GE. Extractions were carried out with a Vibracell 75 115 (Bioblock, Illkirch, France) ultrasonic probe. Alkaline (tetramethylammonium hydroxide, TMAH) digestions of samples and extracts were performed in an Ethos Plus microwave Labstation (Milestone, Sorisole, Italy) fitted with 100-mL closed Teflon vessels, Teflon covers, an HTC adapter plate and HTC safety springs (Milestone). An RM100 agate mortar grinder mill (Retsch, Haan, Germany) equipped with an agate pestle was used to grind dried seaweed samples. Enzymatic digestions were performed at 37 °C on a shaking water bath Grant OLS-200 from Keison Products (Essex, UK). Other material included 1-kDa cutoff membranes (Microsep 1K OMEGA from PALL, Port Washington, NY) for the filtration of the water-soluble fractions and Whatman Chromatography 3 MM paper (Whatman, Kent, UK) for the drying of the gels.

2.2. Reagents and solutions

All reagents were from Sigma-Aldrich-Fluka-Riedel-de Haën (St. Gallen, Switzerland) except: nitric acid (Baker, NJ), tellurium standard solution (Analab, Norwalk, CT), iodide and iodate from Merck (Darmstadt, Germany), rehydration solution, IPG buffer, Strips, oil-dry strip cover fluid-clean up kit, all from GE Health care (Buckinghamshire, UK), and glycerol from VWR, Prolabo (Fontenay-sous-Bois, France). All solutions were prepared in ultrapure water, 18 MΩ cm resistivity, obtained from a Milli-Q purification device (Millipore, Bedford, MA).

Table 1
Chromatographic conditions.

SEC column (separation range)	Mobile phase (flow rate)
HR 10/30 Superdex 75 (3–70 kDa)	30 mM pH 8 Tris–HCl (0.7 mL min^{-1})
HR 10/30 Superdex Peptide (0.1–7 kDa)	100 mM, pH 7.6 ammonium acetate (0.7 mL min^{-1})

LA-ICP/MS was optimized using NIST SRM 612 (glass). Equilibration solution for 2D PAGE consisted of 6 M urea, 29% glycerol, 2% SDS in 75 mM Tris–HCl, pH 8.8. The SDS gel electrophoretic separation was calibrated with a commercial mixture of protein standards (Amersham Low Molecular Weight Calibration Kit for SDS Electrophoresis No. 17-0446-01) from GE Healthcare Life Sciences (Uppsala, Sweden).

2.3. Seaweed sample

Nori (*Porphyra umbilicalis*) edible seaweed, harvested in the Galician coast (northwestern Spain) and commercialized as dehydrated products was studied. Algae leaves were first cut into small pieces and then put into an oven at 40 °C overnight before grinding in an agate mortar and then preserved in polyethylene bottles.

2.4. Procedures

The experimental procedure is schematically outlined in Fig. 1.

2.4.1. Alkaline digestion of samples

Total iodine in samples and pellets was determined as described elsewhere [17]; in brief, ca. 0.1 g of grinded a sample was weighed in a 100-mL Teflon vessel, then 5 mL of water and 5 mL of TMAH were added. A microwave temperature program was set as follows: 10 min ramp from room temperature to 200 °C and 5 min step at 200 °C. After cooling, the samples were centrifuged, transferred to 50 mL volumetric flasks, filtered through 0.45 μm filter and stored in polyethylene bottles until measurement. Similarly, total iodine was quantified in water, NaOH, SDS, Triton X-100, urea and CHAPS extracts [5 mL] after microwave-assisted alkaline digestion with 5 mL of TMAH under the same microwave operating conditions. Blanks were run for every extraction and digestion step.

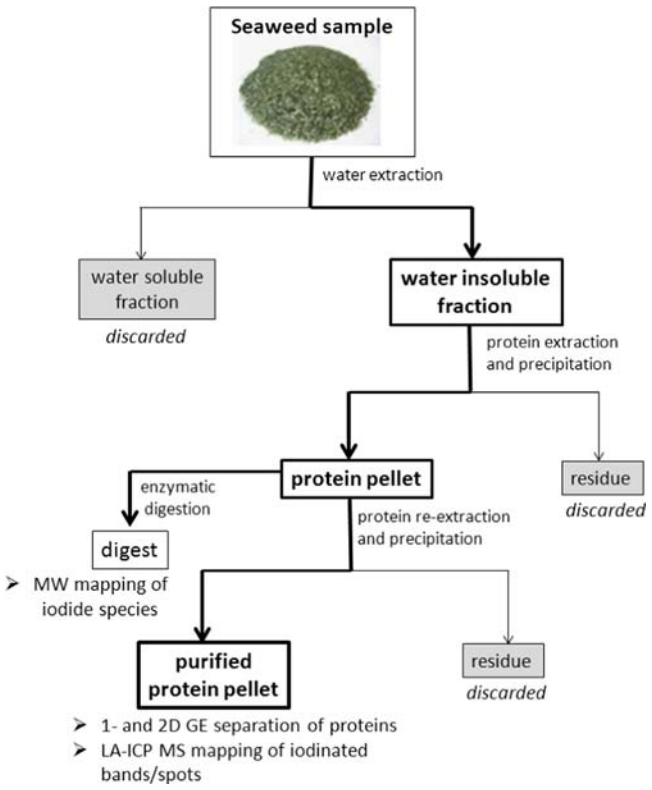

Fig. 1. Experimental procedure.

2.4.2. Separation of water-soluble iodinated compounds

A 0.2 g powdered seaweed sample was weighed in 15 mL tube and extracted by sonication with two 10-mL portions of water in an ice-bath. The ultrasonic probe was set at amplitude of 33% for 1 min, in periods of 2 s sonication followed by 5 s pause. The extracts were centrifuged at 4000 rpm for 10 min and passed through 20 μm filters.

2.4.3. Extraction of water-insoluble iodinated proteins

After the removal of water-soluble compounds, the residual pellets were treated in parallel with: 0.1% SDS, 0.2% Triton X-100, 8 M urea, all of then buffered in 0.1 M Tris–HCl at pH 7.6, and 0.1 M NaOH. Each time 5 mL of the extractant was poured on the pellet and then, 50 μL of a DTT solution (30 mg mL^{-1} in Tris–HCl 0.1 M pH 7.6) was added as reducing agent. The extraction was assisted with an ultrasonic probe set at amplitude of 33% for 1 min, in periods of 3 s sonication and 1 s pause. Supernatants were collected after centrifugation and filtered through 0.45 μm. For protein precipitation, 1 mL aliquot of the extract was mixed with 5 mL of cold acetone and let to stand at −20 °C for 3 h. Pellets were collected after centrifugation and put under a nitrogen flow to eliminate remaining acetone while avoiding complete drying.

2.4.4. Enzymatic digestion of proteins extracted with urea

Pellets obtained after extraction with 8 M urea (as explained in Section 2.4.3) were re-dissolved in 100 μL of 8 M urea (in 100 mM ammonium acetate, pH 8) and transferred to 1.5 mL Eppendorf tubes. Then, 900 μL of a solution, containing 10 mg of either protease XIV or trypsin (prepared in 100 mM ammonium acetate, pH 8) were added. Tubes were shaken in a water bath at 37 °C overnight. Then, the samples were centrifuged and supernatants were filtered through 0.20 μm filter before injection on SEC in a Superdex Peptide column. Blanks were prepared to control possible contamination.

2.4.5. Phenol-based extraction procedure

A 0.2-g of sample was weighed. The water-soluble compounds (WSF) were removed as described above. Then 5 mL of the lysis solution (0.5% CHAPS, 1.5% PVP, 0.7 M sucrose, 0.1 M KCl, 0.25 M EDTA, 2% 2-mercaptoethanol and 34 μL of protease inhibitor cocktail, in 0.5 M Tris–HCl pH 7.5) were added to the water-insoluble fraction. Extraction was assisted with an ultrasonic probe set at amplitude of 33% for 1 min, in periods of 2 s sonication and 5 s pause. After centrifugation, the supernatant was collected and the pellet discarded. Then, the proteins were re-extracted by liquid–liquid extraction with 5 mL of phenol solution (equilibrated with 10 mm Tris–HCl, pH 8.0). Once again, samples were centrifuged and the upper phenol phase was pipetted off carefully while the bottom phase was discarded. Then, aliquots of 2 mL of extract were used for protein precipitation with 8 mL of 0.1 M ammonium acetate in methanol (previously cooled at −20 °C for 1 h) at −20 °C for 3 h. The protein pellets were collected after centrifugation and washed with 7 portions of acetone and finally with the 2-D clean-up kit (GE Healthcare Life Sciences), prior to loading on IEF for 2D gel electrophoresis.

2.4.6. ICP–MS and laser ablation ICP/MS conditions

For quantification of total iodine in solutions resulting from TMAH digestion, the method of standard addition was applied. Tellurium (^{125}Te) was used as an internal standard at a concentration of 50 ng mL^{-1}. ICP–MS conditions (gas flows and RF power) were optimized on a daily base with a 1 ng mL^{-1} of yttrium, thallium and cerium solution (in 2% HNO_3). The details of the experimental conditions are given in Table 2. ^{1}D electropherograms were processed in Microsoft Office Excel 2007; the concentration heatmaps were obtained using a lab-written application for Matlab R2009b (Mathworks, Natics, MA).

2.4.7. Gel electrophoresis conditions

2.4.7.1. 1-D SDS PAGE. A 15-μL aliquot, containing 20–80 μg of proteins, was loaded into each well on top of the gel. For the separation, the voltage was set at 100 V. Once the migration was finished, gels were introduced into a staining solution (30% methanol, 10% acetic acid, 0.1% Coomassie brilliant blue) for 1 h. Gels were then de-stained overnight in a 10% methanol/10% acetic acid solution and dried on Chromatography 3 MM paper at 80 °C.

2.4.7.2. 2D SDS PAGE. Protein pellets containing around 100 μg of proteins were re-dissolved in the rehydration solution containing 2% of IPG buffer. Immobiline™ DryStrip gels were rehydrated during 17 h in the sample solution. IEF was set as follows: 300 V (00:01 h), 3500 V (01:30 h), 3500 V (02:35 h). Then the strips were soaked for 15 min in a DTT solution (50 mg in 5 mL eqilibration solution) and then for 15 min in iodoacetamide solution (125 mg in 5 mL equilibration solution). The second dimension SDS PAGE was performed in 12% polyacrylamide vertical resolving gels at 100 V for the first 20 min and 140 V until the end. Gels were stained/destained and dried as described above. The SDS gel electrophoretic separation was calibrated with a commercial mixture of protein standards (Amersham Low Molecular Weight Calibration Kit for SDS Electrophoresis No. 17-0446-01 from GE Healthcare Life Sciences, Uppsala, Sweden).

3. Results and discussion

3.1. Initial characterization of the algae sample

The sample studied contained 67.1 ± 1.3 μg g^{-1} iodine with 17.1 ± 1.6 μg g^{-1} in the water water-soluble fraction. The molecular size mapping of the water soluble iodine fraction is presented in Supplementary information (Fig. S1). It shows two intense iodide peaks: one eluting after the permeation limit and corresponding to iodide (the identity confirmed by anion-exchange chromatography, results not shown) accompanied by a peak at the exclusion limit (70 kDa). The water soluble fraction was not dealt with furtheron.

3.2. Optimization of extraction of iodinated proteins from algae

Four extractants including NaOH [18], SDS, Triton X-100 and urea were evaluated for the extraction of iodine-containing proteins from the water insoluble fraction from algae. The criterion for this part of the optimization was the extraction yield in terms of the total iodine recovered in the extract. As shown in Table 2, the NaOH solution extracts the highest amount of iodinated compounds. Nevertheless, after precipitation with acetone, the pellet obtained after extraction with urea was that presenting the highest amount of iodine, presumably in the form iodine-containing proteins. The extraction efficiencies of SDS and Triton X-100 were similar and lower than urea and NaOH (Table 3).

3.3. SEC of enzymatic digests

The proteins extracted in the optimized conditions were digested with proteolytic enzymes (protease XIV and trypsin) and the iodinated species present in the digestates were analyzed by size-exclusion chromatography (SEC) – ICP–MS. The chromatograms obtained are presented in Fig. 2. The chromatograms of protease XIV digestate show the presence of iodinated aminoacids (MIT/DIT) thus indirectly confirming the proteinaceous character of the

Table 2
Summary of instrumental and data acquisition conditions.

ICP–MS conditions	
Injector ID (mm)	1.5
Plasma conditions	Wet: 2% HNO_3
Carrier gas flow (He), mL min^{-1}	500
Blend gas (Ar) (mL min^{-1})	740
Cones set	Platinum
RF power (W)	1600
Collision gas	H_2
Collision gas flow (mL min^{-1})	3.6
^{78}Se dwell time (ms)	250
Laser conditions	
Laser source	Nd-YAG
Repetition rate (Hz)	20
Fluence (J/cm^2)	4.1
Spot size/scan width (μm)	250
Scan mode	Line
Scan speed (μm/s)	50 (1D gel), 100 (2D gel)
carrier gas flow (He) (mL min^{-1})	500

Table 3
Evaluation of different solutions in the extraction of water-insoluble iodinated compounds from Nori sample.

Extractant	Concentration of iodine, (μg g^{-1})	
	In the extract	In the precipitate
NaOH	53.5 ± 5.8	6.1 ± 0.8
SDS	13.3 ± 1.7	4.9 ± 1.4
Triton X-100	8.8 ± 0.4	3.2 ± 0.1
Urea	30.1 ± 3.6	11.2 ± 1.0

original species. It is consistent with the recent report on Nori sample showing the presence of both MIT and DIT species [19]. Some residual non-digested species with higher molecular weight could also be observed. Trypsin digestion resulted in a mixture of iodinated species of relatively high molecular mass (ca. several kDa); however, it must be noted that although the MW of these species was within the separation range of the column, the elution some species occurred after the permeation limit, thus demonstrating the presence of non-specific interactions with the stationary phase. This phenomenon is due to the hydrophobicity of some iodinated aminoacids and was observed elsewhere [20]. The tryptic digest chromatogram also shows a peak at the exclusion limit (> 7 kDa), which indicates incompleteness of the digestion.

3.4. Characterization of iodinated proteins by SDS PAGE followed by LA-ICP/MS

After the indirect confirmation of the presence of iodinated proteins in algae, the proteins extracted in the optimized conditions were studied by gel electrophoresis. However, in the first experiments no protein separation was achieved. Only smears were observed (results not shown), presumably due to the insufficient purity of the extract. It was reported elsewhere that GE separation of algae proteins was hampered by the presence of high levels of interfering compounds including polysaccharides or pigments [21–23] and the composition of different algae cells made unlikely finding of an universal protein extraction method [24].

Wong et al. [25] demonstrated the usefulness of a combination of phenol/chloroform extraction method for the red seaweed *Gracilaria changii*. Contreras et al. [26] proposed the combination of a lysis solution and a liquid–liquid extraction with phenol for the proteomic study of different brown seaweed. Nagai et al. [27]

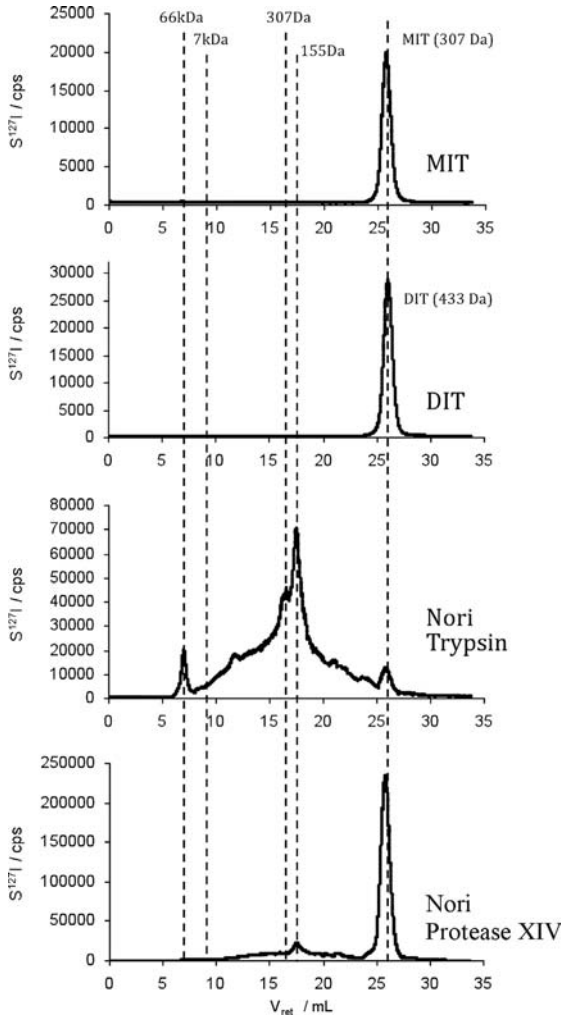

Fig. 2. SEC-ICP–MS of protein enzymatic digests Nori (a,b) standards, (c) digestion with protease XIV and (d) digestion with trypsin.

and Yotsukura et al. [28] developed an ethanol/phenol procedure to carry out the 2D-PAGE of proteins from various brown algae. Recently, Kim et al. [29] optimized an extraction procedure with a lysis solution containing urea, CHAPS (3-[(3–cholamidopropyl) dimethylammonio]-1-propanesulfonate), thiourea, dithiothreitol (DTT), pharmalyte, polyvinylpyrrolidone (PVP), for pre-treating *Saccharina japonica*.

For the purpose of this study and in view of the subsequent GE separation, the extraction methods was re-optimized using a solution containing CHAPS followed by liquid–liquid extraction with phenol (Section 2.4.5). Resolving gels of 8%, 10%, 12% and 15% concentration of polyacrylamide were studied. A good resolution of proteins was observed (results not shown) for gel with polyacrylamide content starting with 10%. For the experiments by LA-ICP/MS, 15% resolving gels were chosen, as the most convenient due to the shortest length. The main problem in iodine ICP–MS determination is the elimination of possible carry-over effects which was addressed by the use of a low-volume ablation cells and optimization of the carrier gas flow.

Fig. 3a shows a LA-ICP–MS 1-D SDS gel electropherogram obtained for the protein extract of *Nori*. It contains several protein bands proving incorporation of iodine in protein bands corresponding to molecular masses of 110 kDa, 40 kDa, 27 kDa, 20 kDa and 10 kDa. A 2D gel electrophoresis with laser ablation ICP–MS ^{127}iodine imaging (Fig. 3b) indicates the presence of iodine in several spots. In rectangle 2, two iodine-containing spots were

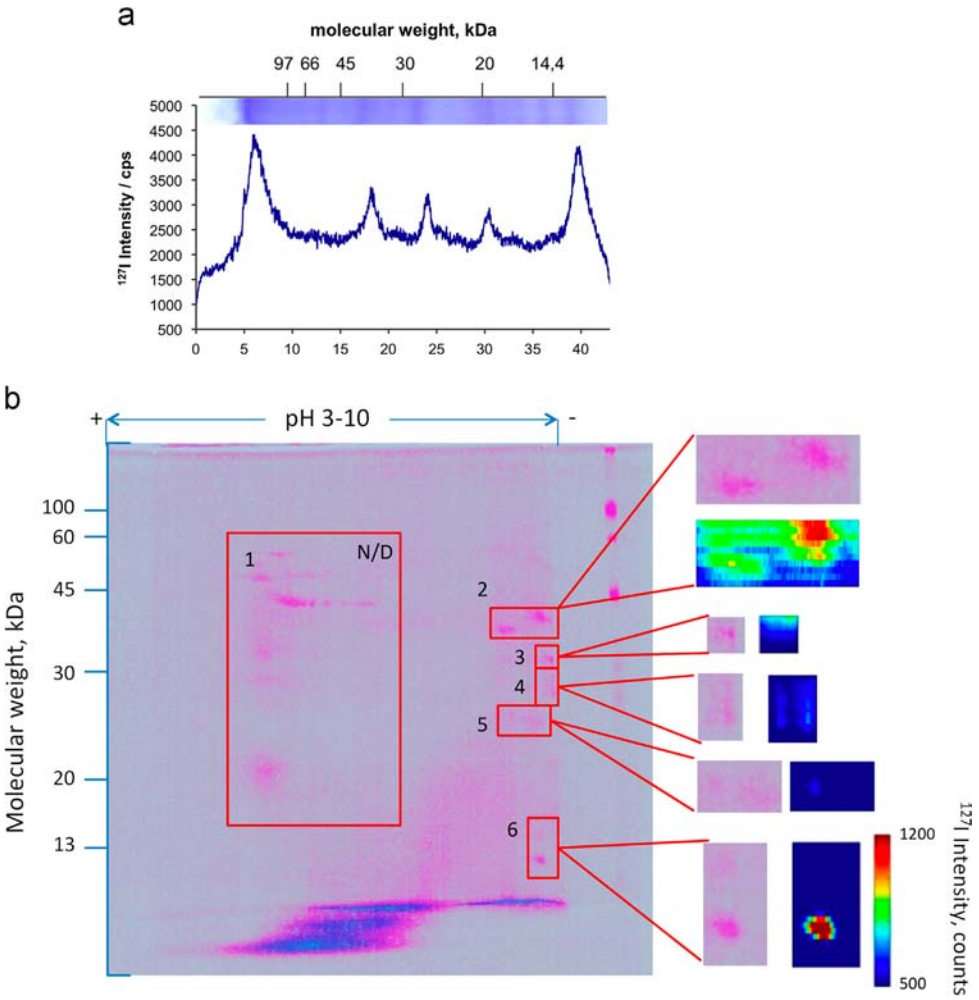

Fig. 3. LA-ICP–MS of SDS PAGE gels obtained for Nori proteins (a) 1D separation and (b) 2D separation.

detected, one at pH∼8.3, ∼35 kDa and another one at pH∼8.8, ∼40 kDa. Rectangle 6 presents the most intense signal at pH 8.8, MW below 13 kDa. Although very low in intensity, two spots were observed in rectangle 4 at around pH 9.1, MW 28 kDa; and one in rectangle 5 at pH 8.3, MW 26 kDa. In rectangle 1 (within pH 4.8–6.6, and MW 60–45 kDa) several protein spots were detected by Coomassie blue staining. However, no iodine was detected by LA-ICP/MS. In rectangle 3, the acquired iodine signal does not clearly correspond to the shape of the protein spots. By comparison with Fig. 3a, it can be assumed that proteins from rectangle 2 correspond to second peak on the electropherogram [MW 40 kDa] while the proteins from the rectangle 4 correspond to the third peak. No clearly defined spots were detected by 2D GE at 20 kDa where the fourth peak on the 1D electropherogram was observed. The most intense iodine spot, in rectangle 6, corresponds to the fifth peak. The absence of spots corresponding to peak 4 in the 1D electropherogram can be explained by a loss during the protein purification step before 2D GE separation, which is more complex in comparison to SDS PAGE separation where crude extract was loaded.

4. Conclusions

1D and 2D gel electrophoresis with laser ablation ICP–MS detection provide first unambiguous evidence on the presence of iodine in algal proteins allowing an insight in their speciation. A careful of optimization of the extraction of iodinated proteins is necessary for successful electrophoresis. The LA-ICP–MS iodine-selective detection demonstrated the presence of iodine in alkaline proteins with molecular masses of 10, 20, 28, 40 and 110 kDa in the alkaline (pH 8–9) p*I* range. The work presents the first ever direct evidence of the presence of iodinated proteins in Nori algae thus opening a way to studies of the potential role of these species for seaweed consumers.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.04.003.

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