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Comparison of different extractive procedures for proteins from the edible seaweeds *Ulva rigida* and *Ulva rotundata*

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Abstract

Proteins have been extracted from the edible seaweeds *Ulva rigida* Agardh and *Ulva rotundata* Bliding using classical or enzymatic procedures. The protocols using NaOH under reductive conditions or a two-phase system (PEG/K_2CO_3) produced the best protein yields. The cleavage or the limitation of the linkages between proteins and polysaccharides caused by these experimental conditions probably explains the efficiency of these protocols. In SDS PAGE, the protein fraction obtained after NaOH extraction from *U. rotundata* is characterised by the presence of three major bands with apparent molecular weights of 45 600, 31 800 and 18 600. The protein fraction from *U. rigida* presents two specific bands with apparent molecular weights of about 27 000 and 12 000. These fractions are mainly rich in aspartic and glutamic acids, alanine, glycine and contain few hydroxyproline residues (0.91–2.44% total amino acid content). The use of cellulase does not significantly improve the extraction of algal proteins in comparison with the blank procedure (without enzymes). The weak accessibility of the substrates in the intact cell wall could explain these experimental data. The improvement of protein yield after the use of the polysaccharidase mixture (β-glucanase, hemicellulase, cellulase) partially confirms this hypothesis.

Introduction

The extraction of algal proteins by classical procedures is hindered by the presence of cell wall mucilage. These anionic or neutral polysaccharides limit the efficiency of the protein extraction. The procedures described in the literature mainly concern the extraction of particular proteins as proteases (Kadokami *et al.*, 1990), peroxidases (Sheffield *et al.*, 1993), carboxylases (Hilditch *et al.*, 1991) or phycobiliproteins (Siegelman *et al.*, 1978).

In comparison, little information about the extraction of the total protein fraction from edible seaweeds is available. Most studies about the nutritional value of algal proteins were performed on the alkali soluble proteins which represent 7 to 20% of total proteins (Fujiwara-Arasaki *et al.*, 1984). The green seaweeds belonging to the genus *Ulva* have been described as a potential source of proteins for human nutrition (Ito & Hori, 1989). The species *Ulva rigida* and *U. rotundata* are widely distributed along the French coasts (Bliding, 1968; Cabioc'h *et al.*, 1992; Gayral & Cosson, 1986) and were recently authorized for human consumption (Fleurence, 1991; Mabeau & Fleurence, 1993).

The present study compares several protein extraction procedures applied to the edible seaweeds *U. rigida* and *U. rotundata*. Among the procedures studied, polysaccharidases action (cellulase, hemicellulase, β-glucanase) was also tested for improving the extraction of proteins. Some biochemical characteristics of the protein fractions obtained by the most efficient procedure were also determined.

Material and methods

Sample preparation

The seaweeds were collected in March 1993 at Pleubian on the Brittany coast. Epiphytes were removed

and the samples were successively rinsed with sea water and distilled water. After this operation the seaweeds were dried by freeze-drying and stored at −20 °C.

Extraction procedures

Each chemical extractive procedure was performed using 10 g algal powder obtained by grinding the dry material with a pestle and a mortar. In the case of enzymatic extractive procedures, the polysaccharidases were applied to 10 g unground freeze dried algae prior to addition of the extraction solvent. Each protocol was repeated 6 times.

Extraction with deionized water (EX1)

The algal powder was suspended in deionized water (200 ml) to allow cell lysis by osmotic shock and facilitate protein extraction. The suspension was gently stirred overnight at 4 °C. After incubation, the suspension was centrifuged at 10 000 × *g* for 20 min and the supernatant collected for the assay of proteins.

Extraction with Tris HCl (0.1 M) buffer (EX2)

The dry material was suspended into 200 ml Tris HCl buffer (0.1 M pH 7.5). This suspension was treated as above (EX1).

Extraction with Tris HCl (0.1 M) buffer with sonication (EX3)

The procedure was similar to EX2, except that suspension was submitted to ultrasound (Ultrasonick 300 Ney, maximal power) for 1 h before stirring overnight at 4 °C.

First extraction with Tris HCl (0.1 M) buffer and second extraction with NaOH (0.1 M) (EX4)

This protocol has been already performed on *Scenedesmus acutus* (Venkarataman & Shivashanka, 1979). The algal powder was initially submitted to the EX2 procedure. The supernatant and the pellet were recovered. The pellet was treated with NaOH (0.1 M) in presence of mercaptoethanol (0.5% v/v). The mixture obtained was gently stirred at room temperature for 1 h before centrifugation (10 000 × *g*, 20 min). The supernatant was collected and combined with the supernatant of the first centrifugation.

First extraction with deionized water and second extraction with NaOH (0.1 M) (EX5)

The first part of the protocol was analogous to the EX1 protocol. The pellet was treated as above (EX4).

Extraction in aqueous polymer two-phase system (PEG/K_2CO_3) (EX6)

In this procedure, anionic polysaccharides present a high affinity for the salt saturated aqueous phase and proteins show affinity for the polyethylene glycol phase. The experimental conditions were identical to those described by Jordan & Vilter (1991).

Extraction with polysaccharidases (EX7, EX8)

Two types of polysaccharidases were tested. The effect of cellulases (cellulase A purchased from Gist-Brocades) on the protein extraction was studied in EX7 and a commercial mixture of polysaccharidases (Rohacent 7005C purchased from Röhm) containing cellulase, hemicellulase and β-glucanase was applied to the algal material in EX8.

Enzymatic solution preparation

The cellulase or polysaccharidase powder (6 g) was added to 200 ml phosphate buffer (0.1 M pH 6) and left under agitation (4 °C) for at least 3 h. The solution was centrifuged at 10 000 × *g* for 20 min at 4 °C. The supernatant was recovered and used as the enzymatic solution. The total activities of the cellulase and the mixed polysaccharidase solutions were determined at 30 °C using carboxymethylcellulose as substrate. The cellulase solution and mixed polysaccharidase solution possessed activities of 18 200 μkatal and 20 000 μkatal, respectively. Before incubation, salts were added (0.5 M NaCl, 40 mM $MgCl_2$, 5 mM KCl) to the enzymatic solution as recommended by Le Gall *et al.* (1990) and Potin *et al.* (1991).

Enzymatic extraction procedure

Enzymatic medium (200 ml) was added to the algal material cut into small pieces (Potin *et al.*, 1991). The suspension at pH 6 was incubated at 30 °C for 2 h. After this time, the algae were filtered through a nylon mesh and the filtrate stored at 4 °C. The residue was ground with a pestle and a mortar in 100 ml Tris HCl (0.1 M pH 7.5) at 4 °C. The homogenate obtained was filtered

and the residue was treated as above. All the Tris HCl filtrates were combined and centrifuged at 10000 × *g* for 20 min at 4 °C. The supernatants were collected and the pellets were eliminated. An incubation without enzymes was also performed as blank procedure.

Protein assay

The amount of proteins were determined by the Kjeldahl method (N × 6.25) (Indergaard & Minsaas, 1991). The values of the proteins contained in the enzymatic powder were subtracted from the protein values determined in the final extract.

Amino acid composition

The proteins were hydrolysed at 110 °C for 24 h with 6N HCl. The amino acid composition was evaluated by HPLC using a cation exchange column (Pickering types 316 SS) and ninhydrin derivation post column. The aqueous alkali fractions (EX5) obtained with the best yield and who apparently contain the same proteins as those other fractions were chosen for this determination. The amino acids analyses were repeated 4 times.

Electrophoresis

SDS PAGE was performed using Laemmli system (Hames, 1981). The acrylamide (Biorad) concentrations were 5% for the stacking gel and 10% for the resolving gel. The protein extracts and a low molecular weight calibration kit (LMW Pharmacia) were allowed to migrate for about 4 h at 35 mA. The proteins were revealed by Coomassie Blue staining.

Results

The levels of total protein are similar for the two species (10.1 and 11.2% of dry weight) (Table 1). The simple extraction procedure using deionized water (EX1) gives a low protein yield for both species (Table 2). Tris HCl buffer with (EX3) or without the use of ultrasound (EX2) does not significantly improve this yield (Table 2). However, the aqueous two phase extraction system (EX6) significantly increases the recovery of proteins (2.0 fold for *U. rigida*, 2.3 fold for *U. rotundata*) (Table 2). The treatment of the pellet obtained after Tris HCl extraction by NaOH and mercaptoethanol (EX4) also significantly improves the yield (Table 2). However, the highest protein yield (Table 2) is observed after the application of the procedure which uses NaOH and mercaptoethanol after extraction with deionized water (EX5). This latest protocol permits a threefold increase in protein extraction from *U. rigida* as compared to the simple aqueous procedure (EX1) (Table 2). It also increases the protein yield about 2.6 fold from *U. rotundata* (Table 2). No major difference was observed for either seaweed in SDS protein patterns obtained from different procedures (data not shown). Notably, the major bands (Fig. 1) are present in all SDS patterns. On the other hand, not any protein bands are detected in SDS gels with the extracts containing polyethylene glycol (EX6) and patterns after enzymatic procedures are too complex for a suitable interpretation.

Aspartic and glutamic acids constitute the main amino acids (Table 3) in alkali soluble fractions of *U. rigida* and *U. rotundata*. However, *U. rigida* samples can be distinguished from *U. rotundata* samples by lower alanine and glycine contents (Table 3). Small quantities of hydroxyproline (0.91% to 2.44% total

Table 1. Dry matter and protein contents of *Ulva rigida* and *U. rotundata* samples.

	Ulva rigida	*Ulva rotundata*
Dry matter (103 °C) (as % fresh weight)	13.8 ± 0.6	13.9 ±0.4
Protein (as g kg^{-1} dry weight)	112.0 ± 5.8	100.1 ± 4.9

Table 2. Influence of extraction procedures on protein yield from *Ulva rigida* and *Ulva rotundata*. Results expressed as % of total protein (Proteins extracted/Total proteins × 100).

Extraction procedure	Proteins extracted from total proteins of *Ulva rigida* (%)	Proteins extracted from total proteins of *Ulva rotundata* (%)
EX1	9.73 ± 0.6	14.00 ± 1.8
EX2	9.37 ± 1.6	13.80 ± 1.2
EX3	10.35 ± 0.8	16.08 ± 0.9
EX4	17.50 ± 1.3	25.17 ± 1.9
EX5	26.80 ± 1.3	36.10 ± 1.4
EX6	19.10 ± 1.1	31.56 ± 2.1
EX7	<1.00	<1.00
EX8	18.48 ± 2.1	22.00 ± 1.5

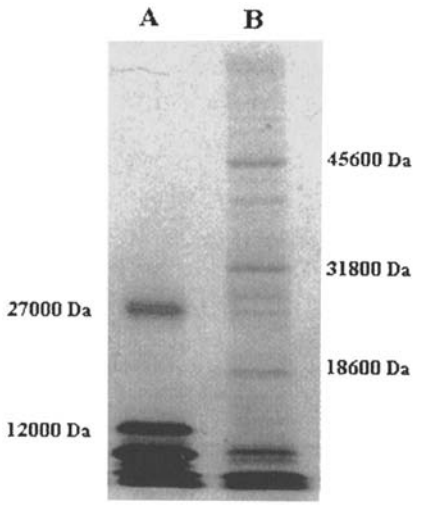

Fig. 1. Electrophoretic patterns (PAGE SDS) of alkaline protein fractions obtained from *Ulva rigida* (A) and *Ulva rotundata* (B). Coomassie Blue Staining.

amino acids fraction) (Table 3) are also detected in both protein fractions and *U. rotundata* samples show a higher level of hydroxyproline than *U. rigida* samples (Table 3). Alkali fraction obtained from *U. rotundata* samples is characterized in SDS PAGE by the presence of three major bands with molecular weights of about 45 600, 31 800 and 18 600 respectively (Fig. 1). *U. rigida* possesses two major bands with an apparent molecular weight of 27 000 and 12 000 (Fig. 1). No significant effect on protein extraction is observed with the use of the cellulase solution (EX7) (Table 2). On the other hand, the use of the polysaccharidase mixture (EX8) gives an extraction yield close to those observed with the procedure using Tris HCl buffer and NaOH (EX4) (Table 2). However, the enzymatic procedures appear to be less efficient than procedures using deionized water and NaOH (EX5) (Table 2).

Table 3. Amino acid composition of protein fractions obtained after NaOH extraction (EX5).

Amino acid	A.A. as mg g^{-1}	A.A. as % total amino acid fraction
Ulva rigida		
Aspartic acid	20.76	14.74
Glutamic acid	15.86	11.27
Alanine	10.26	7.29
Valine	9.80	6.96
Threonine	9.63	6.84
Glycine	9.51	6.75
Leucine	9.34	6.63
Arginine	8.49	6.88
Serine	7.69	5.46
Proline	6.50	4.62
Isoleucine	6.29	4.46
Tyrosine	5.86	4.16
Lysine	5.57	3.96
Phenylalanine	4.94	3.51
Methionine	2.97	2.11
Histidine	2.87	2.04
Hydroxyproline	1.28	0.91
Cystein	0	0
Ulva rotundata		
Glutamic acid	30.53	16.49
Aspartic acid	27.76	14.99
Alanine	15.02	8.11
Glycine	13.65	7.87
Leucine	11.47	6.19
Valine	11.22	5.84
Threonine	9.63	6.06
Serine	9.39	5.07
Lysine	8.99	4.86
Arginine	8.59	4.64
Isoleucine	7.58	4.10
Tyrosine	6.61	3.46
Phenylalanine	6.41	3.57
Hydroxyproline	4.52	2.44
Methionine	4.45	2.41
Histidine	2.81	1.52
Cystein	0	0

Discussion

The protein values determined from *U. rigida* and *U. rotundata* samples are within the range generally observed (10–20% dry weight) for the seaweeds collected at the Pleubian site (CEVA, unpublished data). However, they are lower than those recorded in the literature for *Ulva* sp. (Indergaard & Minsaas, 1991) and *U. pertusa* (Fujiwari-Arasaki *et al.*, 1984). Like *Laminaria digitata* (Jordan & Vilter, 1991), the use an aqueous two-phase system significantly improves the extraction yield. The limitation in solution of interactions between polysaccharides and proteins probably explains this fact. However, the best yield is obtained with extraction in basic medium contain-

ing β-mercaptoethanol. This result agrees with data observed for *Ulva* sp. (Serot *et al.*, 1994) and *S. acutus* (Venkarataman & Shivashanka, 1979). However, the denaturation effect of NaOH and mercaptoethanol on the tertiary structure of proteins suggests a limited and controlled use of this protocol. The lack of major differences between the SDS patterns obtained from the different extracts suggests that the different protocols allow the extraction of the same proteins. The presence of aspartic and glutamic acids as the main animo acids in alkali fractions of both *U. rigida* and *U. rotundata* samples confirms the results recorded for *Ulva* sp. (Indergaard & Minsaas, 1991) and *U. pertusa* (Fujiwara-Arasaki *et al.*, 1984). The presence of hydroxyproline in the protein fraction of *U. rotundata* and *U. rigida* agrees with data reported from other green algae (Thompson & Preston, 1967). This result suggests the presence of "extensin like" proteins in the *Ulva* species. The electrophoresis pattern of *U. rigida* samples show two characteristic major bands with apparent molecular weights close to those recorded for *Ulva* sp. (Serot *et al.*, 1994) and *U. lactuca* (Ochiai *et al.*, 1987). On the other hand, the patterns of *U. rigida* and *U. rotundata* samples are very different (Fig. 1). A more comprehensive study, including samples collected in different seasons and sites, is necessary to conclude on the use of SDS PAGE as a tool for *Ulva* species identification.

The lack of effect of cellulase solution on the extraction procedure could be explained by low accessibility to the substrate and by the conditions applied (e.g. a pH value relatively far from optimum pH of cellulase). However, in our case, the pH conditions used for the cellulase action are similar than those described for optimal production of *U. rotundata* protoplasts (Cachot *et al.*, 1994). The access of cellulase to the substrate probably requires a partial destruction of the cell wall by the action of other enzymatic activities. This hypothesis is based on the results obtained after the use of the mixed polysaccharidase solution, which significantly improves the yield. Although less efficient than extraction in aqueous medium containing NaOH and mercaptoethanol, this latest enzymatic procedure could be applied preferentially for the extraction of proteins for which conservation of biological activities or functional properties is necessary.

This study shows that the extraction in basic medium with NaOH and mercaptoethanol allows an optimal recovery of proteins from both *Ulva rotundata* and *Ulva rigida*. The cleavage or limitation of linkages between polysaccharides and proteins appears to be a determinant factor for extraction improvement. These results confirm other experimental data obtained from *Ulva* sp. (Serot *et al.*, 1994) and other algal species (Venkataraman & Shivashankar, 1979; Jordan & Vilter, 1991), that *U. rotundata* and *U. rigida* can be distinguished by the electrophoretic patterns obtained from alkaline fractions. Confirmation of this fact would be of interest for systematic identification of these species. The presence of hydroxyproline in protein fractions obtained after alkaline treatment suggests the presence of this amino acid residue in cell wall proteins such as those identified in *Codium* sp. and terrestral plants (Lamport, 1969). The extractive enzymatic methods appear to present little interest for increase protein extraction yields for two seaweeds studied. However, not all factors which can influence this type of procedure have yet been fully tested. The application of this type of procedure is suggested for the extraction of particular proteins needing mild conditions for conservation of biological properties.

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