Review

Macroalgae as Protein Sources—A Review on Protein Bioactivity, Extraction, Purification and Characterization

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Abstract: The increased demand for protein sources combined with a decrease in the available land and water resources have led to a growing interest in macroalgae as alternative protein sources. This review focuses on strategies for macroalgae protein extraction, enrichment and characterization. To date, the protein extraction methods applied to algae include enzymatic hydrolysis, physical processes and chemical extraction. Novel methods, such as pulsed electric field, microwave-assisted, pressurized liquid and supercritical fluid extractions, and the application of smart solvents are discussed. An overview of the use of membranes and other processes to generate high-value protein concentrates from algae extracts is also presented, as well as some examples of the methods used for their characterization. The potential bioactivities from macroalgae-derived proteins and peptides, including novel glycoproteins and lectins, are briefly reviewed.

Keywords: macroalgae; seaweed; protein extraction; bioactivity

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1. Introduction

A “Biorefinery” is defined as a “sustainable and synergetic processing of biomass into marketable food and feed ingredients, products (chemicals and materials) and energy” by the International Energy Agency (IEA) [1]. Different strategies can be integrated to maximize the extraction of valuable components, with subsequent use of the remaining fractions as raw materials for further processing in a cascading approach, while minimizing waste streams. This concept applied to macroalgae adds value to these bioresources and contributes to sustainable development. It is therefore crucial to choose the best available technologies for an efficient sequential separation of valuable seaweed fractions, in particular of seaweed proteins. Seaweed proteins/peptides due to their availability, amino acid composition and bioactive properties constitute a good option to fulfill the increased demand for protein sources. This review records the available technologies for extraction and enrichment of macroalgal proteins, as well as the methods for their characterization.

1.1. Macroalgae Background

The term algae refers to a large diversity of unrelated phylogenetic entities, ranging from picoplanktonic cells to macroalgal kelps [2]. In contrast to terrestrial plants that share a common ancestor, algal diversity includes several distantly related groups of mainly photoautotrophic organisms from aquatic habitats. The high diversity of macroalgae at high taxonomic levels and related long evolutionary history displayed by complex phylogeographies are reflected in the observed richness and diversity of algal components. Algal chemistry is largely linked to evolution, but phenotypic modifications can also be a

result of environmental and biological stimuli as individual populations exhibit phenotypic plasticity and adaptation to their environment. Because they often experience stressful conditions and highly fluctuating environments, most algae possess mechanisms to enable acclimation to stressors (e.g., UV radiation, temperature and salinity) and are able to defend themselves against biological pressures (e.g., competitors, grazers and/or parasites). This wide range of tolerance combined with their specific cellular structure predisposes them to growth and development under both laboratory and industrial conditions [2].

Macroalgae are multicellular algae with thalli-like structures that can adhere to solid underwater surfaces or float freely in water. They inhabit ecosystems with varied salinities, from freshwater rivers, ponds and lakes to brackish estuaries and the open sea and seashores. Compared to other algal groups, such as microalgae, they occupy available space slower, but grow faster and are less vulnerable to grazing and water turbulence [3]. Macroalgae are classified as green, red and brown algae, according to the thallus color derived from natural pigments. Green algae belong to the *Chlorophyta* phylum and have the same ratio of chlorophyll *a* to *b* as terrestrial plants. There are about 4500 species of green algae [4]. Red algae are included in a single class, *Rhodophyceae*, which consists of two subclasses: *Florideophycidae* and *Bangiophycidae*. The red color is attributed to the pigments chlorophyll *a*, phycoerythrin and phycocyanin. There are about 4000–6000 red algae species in over 600 genera [5]. Brown algae are classified as *Phaeophyceae* and their principal photosynthetic pigments are chlorophyll *a* and *c*, β -carotene and other xanthophylls. Circa 1500–2000 species of brown algae have been identified so far [6]. Marine macroalgae (seaweeds) are vertically distributed from the upper zone (sea surface) to the lower sublittoral zone [7]. In fact, although many species thrive in littoral zones, some red algae inhabit deeper sea areas (over 25 m below the surface) whereas others are found under rocks, where direct sunlight availability is limited [8]. This is because red algae use a different part of the light spectrum from that used by other algae and thus are able to grow in places where the other algae cannot grow.

Macroalgae are photoautotrophic and therefore use photosynthesis to produce and store organic carbon by using CO_2 or HCO_3^- [9]. The photosynthetic rates of macroalgae highly depend on the species although green and red algae usually have higher rates than their brown counterparts (by 1 or 2 orders of magnitude) [3]. When comparing macroalgae and terrestrial plants, significant differences in the chemical composition can be pinpointed in addition to the physiological and morphological ones. Macroalgae contain the typical polysaccharides, such as mannan, ulvan, carrageenan, agar, laminarin, alginate and fucoidan, which are not found in lignocellulosic terrestrial biomass [5]. Like terrestrial plant biomass, macroalgae generally do not possess high contents of starch and lipids, except for green algae (1–4% for starch and 0–6% for lipids). Their lack of lignin makes them less rigid and contributes to the flexibility of their cell wall.

Carbohydrate compounds are abundant in macroalgae. Their contents in green, red and brown algae vary between 25% and 50%, 30% and 60%, and 30% and 50% of their dry weight (dw), respectively [10]. Their composition evidently differs between species. While cellulose is a structural polysaccharide (exists in the cell wall) common to all macroalgae, each group has other typical structural and storage polysaccharides [11]. As the structural polysaccharides, green macroalgae features ulvan [12], while red algae contain either carrageenan (up to 75% dw) or agar (up to 52% dw) [13]. When it comes to brown algae, their major polysaccharide is alginic acid (i.e., alginate). As storage polysaccharides, green and red algae contain starch (floridan starch in the case of red algae) while brown algae have laminarin (β -1,3-glucans), which accounts for up to 35% dw.

It is relevant to note that the composition of macroalgae of the same species can also differ. Local conditions at the collection site, such as light, salinity, nutrients, temperature, pollution and water motion, can considerably influence the metabolite levels and bioactive compound contents. The biological status of the algae (e.g., life cycle, development stage and thallus structure) can also have an impact on their biochemical composition [14].

1.2. Proteins in Seaweed

Proteins from macroalgae of marine origin have been the focus of several studies due to their potential bioactivity. The protein content varies according to phylum. Brown algae generally have a low protein content (3 to 15% dw), which contrasts with the protein content of green (9 to 26% dw) and red algae (20 to 47% dw) [15–17]. A recent review lists the protein content of macroalgae species from different geographical regions [18]. In the latter phylum, it is worthy to highlight the species belonging to the genus *Porphyra*, where the protein content can reach 47% dw [19]. These concentrations are comparable to those found in high-protein vegetables, namely (% dw), leafy greens and legumes (mint—30.9; cilantro—22.2; spinach—26.5; cauliflower—29.9; soybean—35 to 40; chickpea—20 to 25) and major cereals (wheat—8 to 15; barley—8 to 15; rice—7 to 9; corn—9 to 12) [20].

The protein content of macroalgae varies within seasonal cycles. One example is the protein content of the red seaweed *Palmaria palmata* collected at the French Atlantic coast, which showed fluctuations between 9 and 25% dw in protein content. The highest values occurred during winter and spring [15]. Seaweed may contain non-proteinic nitrogen, from nitrates, pigments or nucleic acids, which results in an overestimation of their protein content (usually estimated by the Kjeldahl method, with a general Nitrogen-to-Protein conversion factor of 6.25). Specific Seaweed-Nitrogen-to-Protein (SNP) conversion factors for brown, red and green seaweeds have been reported [21]. Detailed essential amino acids (EAA) and non-EAA profiles, total protein content and protein/non-protein nitrogen amounts of four macroalgae species obtained from an integrated multi-trophic aquaculture system in Ria de Aveiro (from ALGAplus Ltd., Ílhavo, Portugal), have been reported [22].

1.2.1. Phycobiliproteins

Protein–pigment complexes such as the phycobiliproteins (PBPs) are one of the most important groups of marine proteins. In red seaweed, these complexes are the main light-harvesting pigments and account for up to 50% of the total protein content [23]. Phycobiliproteins are the only water-soluble algal pigments. They are a family of fluorescent proteins covalently linked to tetrapyrrole groups, known as bilins. They constitute a structure attached to the cytoplasmic surface of thylakoid membranes named phycobilisomes (unlike carotenoids and chlorophylls that are located in the lipid bilayer) [23]. PBPs are grouped into four classes: phycoerythrin (PE), phycocyanin (PC), phycoerythrocyanins (PEC) and allophycocyanin (APC) [14]. The most common phycobiliprotein in many red seaweeds is known as R-phycoerythrin (R-PE). Isolation of PE has been reported from many species, for instance, *Gelidium pusillum* [24], *Grateloupia turuturu* [25,26] and *Rhodomenia pseudopalmeta* [27].

1.2.2. Glycoproteins and Lectins

Glycoproteins (GPs) are carbohydrate-binding proteins. Glycans can be conjugated to peptide chains by *N*-glycosyl linkages and/or *O*-glycosyl linkages. Protein glycosylation can happen co- or post-translation. GPs are located in the cell wall and on cell surfaces or are secreted [14]. A few seaweed glycoproteins have been isolated by hot- or cold-water extraction. GP-rich fractions obtained from *Ulva* spp. showed a higher content in proteins than in neutral sugars [28]. Extracts of *Ulva lactuca* with high contents of both carbohydrates and protein suggested the presence of GPs [29]. Yoshiie et al. (2012) [30] have performed the structural analysis of the *N*-glycans expressed in several seaweed species. The study revealed that only high-mannose glycans were present in the 15 macroalgae addressed (red, brown and green), while the complex type of *N*-glycans containing β 1–2 xylosyl and α 1–3 fucosyl residues, which is encountered in terrestrial plants, was not found. The same authors discovered that the M9 structure is predominant in the *N*-glycans of the GPs of *Zoostera marina* and *Sargassum fulvellum*. Therefore, they claim the interest of both species as good sources of *N*-glycans used, e.g., as chaperone-like oligosaccharides in protein assembly [31].

Phycolectins are a group of carbohydrate-binding proteins in macroalgae. Lectins interact with specific glycan structures that constitute the soluble and membrane-bound glycoconjugates [14]. Seaweeds are particularly good sources of novel lectins. A few examples are griffithsin, a mannose-specific lectin isolated from the red algae *Griffithsia* spp. [32]; SfL1 and SfL2 from *Solieria filiformis* [33]; and HRL40 from *Halimeda renschii* [34]. Although macroalgae phycolectins have been characterized, very little is currently known about their structural and biochemical properties [14].

Arabinogalactan proteins, which belong to the hydroxyproline-rich glycoproteins, have been reported in the cell wall of few species of seaweeds, namely, in the green seaweeds *Codium vermilara* [35] and *C. fragile* [36].

1.2.3. Enzymes

Seaweeds are rich sources of enzymes. Alkaline phosphatase (Zn-containing metalloproteinase that catalyzes the non-specific hydrolysis of phosphate monoesters) is widely encountered in seaweeds, namely, in *Ulva pertusa* [37]. Alternative oxidases (proteins involved in the electron flow through the electron transport chain and in the regulation of the mitochondrial retrograde signaling pathway) have also been described, namely, from *Caulerpa cylindracea* [38]. The fibrinolytic enzyme (trypsin-like serine protease) has also been isolated from green algae, such as *Codium fragile* and *Codium latum* [39,40]. Rubisco, a protein that catalyzes carbon dioxide fixation and oxygenation, has been obtained from *Kappaphycus alvarezii* [41].

1.2.4. Peptides and Amino Acids

Although the structure and biological properties of algal proteins are still relatively poorly documented, the amino acid composition of several species of macroalgae is known [42]. Most species contain all the EAA for humans, which may represent about 50% of the total amino acids. Besides, their proteins are also rich in aspartic and glutamic acid residues (22.7 g/100 g protein in several red algae) [23]. The levels of some amino acid residues, such as threonine, lysine, tryptophan, cysteine, methionine and histidine, are higher than those found in terrestrial plants [18]. Seaweed amino acid analyses have demonstrated profiles similar to those of ovalbumin (52.4% EAA) and leguminous plants (41.62% EAA) [15,43]. At this point, it is worth mentioning that the interest in marine proteins may not be directly linked to the proteins themselves, but rather to the bioactivity potential of the peptides that can be derived from them [44,45]. Bioactive peptides usually contain 3–40 amino acid residues, and their activities stem from both their amino acid composition and sequence. They are generated from parent proteins either through digestion processes in the gastro-intestinal tract or produced through fermentation or other processes involving enzymatic hydrolysis [46,47].

The free amino acid fraction of macroalgae is composed primarily of alanine, taurine, omithine, citrulline and hydroxyproline. Laminine, kanoid amino acids and mycosporine-like amino acids have also been found in marine macroalgae [48]. The successful production of bioactive peptides that originated from hydrolyzed proteins of *Palmaria palmata*, *Solieria chordalis*, *Ulva lactuca* and *Saccharina longicuris* has been reported [49].

1.3. Applications of Macroalgal Proteins and their Derivatives

Marine macroalgae are rich sources of structurally diverse bioactive compounds with valuable pharmaceutical and biomedical properties. They can also be used as functional health-promoting ingredients in food and feed [50,51]. Besides the benefits, attention must be given to the potential deleterious effects of food and feed derived from contaminated seaweed and to the legislation regulating these applications [51]. The functional properties of proteins are mainly associated with their ability to form and/or stabilize gels and films, foams, emulsions and sols [52]. Macroalgae or macroalgal extracts have shown effects on the immune status and intestinal health of several monogastric farm animal species, including pigs [53], broiler chicken [54] and fish [55]. These bioactive properties could be related to the presence of specific proteins and/or peptides.

1.3.1. Bioactive Proteins

Among all the macroalgal proteins, lectins and phycobiliproteins have received particular attention due to their reported biological activities [42]. The main biological activity associated with lectins is their hemagglutinating activity against erythrocytes [42], whereas phycobiliproteins are used in biomolecule labeling for fluorescent immunoassays, immunohistochemistry assays and fluorescence microscopy, and as natural colorants for food and cosmetic applications [56]. Different phycobiliproteins have exhibited antioxidant, anti-inflammatory, neuroprotective, hypocholesterolemic, hepatoprotective, antiviral, antitumor, liver-protecting, atherosclerosis treatment, serum-lipid-reducing and lipase-inhibition activities [56]. The bioactivities of several macroalgal proteins are shown in Table 1.

Table 1. The bioactivities of several macroalgal proteins.

Species	Type of Protein	Bioactive Activity	Ref.
<i>Bryothamnion triquetrum</i> , <i>Bryothamnion seaforthii</i> , and <i>Amansia multifida</i>	Lectins	Antinociceptive effects.	[42]
<i>Codium fragile</i>	Lectins	Blood typing; characterization of cell-surface polysaccharides; lectinosorbent assays for cell-binding-pattern examinations.	[57]
<i>Codium intricatum</i> , <i>Codium latum</i> , and <i>Codium divaricatum</i>	Fibrinolytic enzymes	Preferentially hydrolyzing fibrinogen Aa chain.	[57]
<i>Eucheuma serra</i>	ESA-2 (lectin)	Colonic carcinogenesis suppression in mice; growth inhibition of 35 human cancer cell lines.	[42]
<i>Eucheuma serra</i> and <i>Galaxaura marginata</i>	Lectins	Antibacterial activity against the fish pathogen <i>Vibrio vulnificus</i> .	[57]
<i>Griffithsia</i> spp.	Novel lectin	Strong anti-HIV activity.	[42]
<i>Hypnea cervicornis</i> and <i>Solieria robusta</i>	Lectins	Anti-inflammatory and mitogenic activities in mice spleen lymphocytes; growth inhibition of mice leukemia cells L1210 and mice FM3A tumor cells.	[42]
<i>Hypnea cervicornis</i>	Mucin-binding agglutinin	Antinociceptive and anti-inflammatory activity.	[42]

1.3.2. Bioactive Peptides

Bioactive peptides have been shown to possess properties such as opioid, immunomodulatory, antibacterial, antithrombotic and antihypertensive activity [58]; some may also exhibit multifunctional bioactivities (Table 2) [59].

An extensive list of bioactivities of the peptides derived from specified seaweeds, including methodologies for their enzymatic production conditions, respective bioactivity evaluation assays and citations, can be found in [18].

Table 2. The bioactivities of several macroalgal peptides.

Species	Peptide	Bioactive Activity	Ref.
<i>Bryopsis</i> spp.	Kahalalide F	Antitumoral activity	[57]
<i>Galaxaura filamentous</i>	Galaxamide	Anti-proliferative activity against human epithelial cancer cell lines	[60]
<i>Palmaria palmata</i>	Asn-Ile-Gly-Gln	Anti-inflammatory activity	[61]
	Val-Tyr-Arg-Thr; Leu-Asp-Tyr; Leu-Arg-Tyr; Phe-Glu-Gln-Trp-Ala-Ser		[62]
<i>Porphyra yezoensis</i>	Ile-Tyr; Met-Lys-Tyr; Ala-Lys-Tyr-Ser-Tyr; Ley-Arg-Tyr	ACE-I inhibitory activity	[63]
	Ala-Lys-Tyr-Ser-Tyr		[64]
<i>Undaria pinnatifida</i>	Val-Tyr; Ile-Tyr; Phe-Tyr; Ile-Trp		[65]
	Val-Tyr; Ile-Tyr; Ala-Trp; Phe-Tyr; Val-Trp; Ile-Trp; Leu-Trp	Anti-hypertensive	

2. Protein Extraction

2.1. Challenges and Impact of Cell Structures

The successful extraction of macroalgal proteins highly depends on their accessibility since most of them are produced intracellularly. Therefore, the complex nature of algal cell walls is the main challenge for the use of seaweed as protein sources. Algal cells possess many intracellular enzymes and proteins [66] and their cell wall is composed of a highly integrated network of biopolymers, mainly polysaccharides, which interact with water and metal cations, amongst other molecules [67]. The cell wall can be divided into three main domains: the fibrillar wall, the amorphous matrix and the glycoprotein domain [23]. The fibrillar polysaccharides and the glycoproteins form a reticulated network that is embedded in the gel-like amorphous matrix. The fibrous part is the most chemically inert and mechanically resistant cell wall component, with cellulose being the most significant compound amongst others such as xylan and hemicellulose. Very little is known about the glycoprotein domain, which is constituted by glycoproteins that contain cellulose-binding domains. The gel-like matrix is made of carboxylic and/or sulfated polysaccharides, like sulfated galactans such as carrageenans and agarans, and usually extends into the intercellular spaces between adjacent cells [23]. Other biopolymers, such as proteins and polymeric phenolics, can also participate in cell wall formation.

The presence of polysaccharide-bound cell wall mucilage, including anionic or neutral polysaccharides, and polyphenols reduce the protein extractability and requires additional steps for fractionation and purification. Polysaccharides induce strong electrostatic interactions [68], whereas polyphenols may form reversible hydrogen bonds with proteins or undergo oxidation. Oxidized phenolic compounds can react with amino acids and form insoluble complexes [69].

The morphology of different seaweed species has also been suggested to be an important factor in protein extraction, with tougher thallus forms reported to require increased processing. The raw biomass from seaweed after harvesting must be preserved by drying or freezing or used fresh as soon as possible to avoid protein degradation [14]. The

increased protein extractability from oven-dried biomass was suggested to have been due to the decomposition of the phenolic compounds, as well as increased disruption of the anionic or neutral polysaccharides found within the cell wall of the seaweed [46].

The combination of extraction methods and purification techniques is necessary to improve the protein yield. The greater the scale, the bigger the challenge because methods with low time, cost and energy consumption (i.e., environmentally friendly) are required.

2.2. Conventional Extraction Methods

As mentioned above (Section 2.1), the extraction and utilization of algal proteins evidently depends on the disruption of the cell wall. To ensure extraction of intracellular proteins, additional stress factors are often applied, which improve the extraction efficiency.

So far, algal proteins have not been fully described, and hence they are simply divided into four main classes based on their solubility, namely, albumins, which are soluble in water; globulins, which are soluble in salt solutions; glutelins, which are soluble in dilute acids or bases; and prolamins, which are soluble in 70% alcohol in water [66]. Sequential extraction steps are often carried out to ensure the extraction of different types of proteins, unless a specific type is being targeted.

Algal proteins are conventionally extracted by means of aqueous, acid, and alkaline solution-based methods, followed by centrifugation for extract clarification and fractionation and enrichment techniques, such as ultrafiltration, precipitation and/or chromatography [70]. Aiming at the preservation of protein structure and affinity interactions ability, mild extraction conditions at defined pH ranges may be required [33,71–73].

Physical cell disruption methods, such as osmotic shock, freeze/thawing, shearing or grinding, can enhance the extraction of protein from some seaweeds. The conventional disruption pre-treatments and protein extraction methods are presented in Table 3.

2.3. Enzyme-Assisted Extraction

Enzyme-Assisted Extraction (EAE) is often the preferable method to extract proteins and/or their hydrolysates from seaweed [46]. Polysaccharidases can be applied as a cell disruption treatment prior to protein extraction to increase the protein yield. Several polysaccharidases (κ -carrageenase, β -agarase, xylanase and cellulase) have been used in protein extractions from red seaweed species, namely, *Chondrus crispus*, *Gracilaria verrucosa* and *Palmaria palmata* [74]. The use of cocktails that contain multiple hydrolytic activities (cellulase, hemicellulase and β -glucosidase) for carrageenan, agar, alginate and cellulose is a promising option to increase the extraction yields [75]. The selective degradation of structural proteins, such as glycoproteins of the cell wall, might be possible by using subtilases or serine proteases [76]. Different digestion enzymes have also been used to release bioactive peptides from parent proteins, with chymotrypsin, trypsin and pepsin being the most used [14]. Combining EAE with other processes (e.g., enzymatic hydrolysis combined with alkaline extraction) is usually the go-to approach (Table 3) [77].

Once again, the enzyme choice is highly correlated with the desired end product (e.g., intact proteins, specific proteins or bioactive peptides). Regarding feasibility, every case needs to be looked at individually. If a high enzyme:substrate ratio is required, an enzymatic treatment might not be viable at the industrial scale, particularly due to the cost of the enzymes.

2.4. Ultrasound-Assisted Extraction

Ultrasound-Assisted Extraction (UAE) has been shown to be an attractive technology when used on cellular matrices. It acts by creating compression and decompression through sound waves at frequencies higher than 20 kHz. UAE can be applied to numerous food sources, particularly in the modification of plant micronutrients to improve the bioavailability, simultaneous extraction and encapsulation, quenching radical sonochemistry to avoid degradation of the bioactive compounds, as well as to increase the bioactivity of the phenolics and carotenoids by targeted hydroxylation [78]. The bioavailability of seaweed proteins can also be improved by the degradative effect of sonochemistry, which is not produced by the ultrasound waves directly but rather by acoustic cavitation. This happens when the static pressure falls below the vapor pressure of the liquid and the formation and growth of vapor bubbles occurs. Such bubbles, under the subsequent pressure peak, violently collapse, leading to peeling, erosion, particle breakdown and degradation of the solid–liquid surfaces. Solvent penetration into the cells is facilitated, as is the release of the intracellular compounds to the bulk solvent [79].

The application of ultrasound can be divided into two different categories, namely, low intensity–high frequency (100 kHz–1 MHz) and high intensity–low frequency (between 20 and 100 kHz) ultrasound, the latter being the type that is typically used for the disruption of cell walls and membranes [80]. Higher extraction yields are usually achieved at shorter processing times and lower temperatures, making this method suitable for the extraction of thermolabile compounds [81]. Under these conditions, solvent consumption is also lower, facilitating the downstream processing of the target compounds [82].

An ultrasound pre-treatment reportedly increased protein extraction from *Ascophyllum nodosum* by 540% and 27% when followed by acid or alkaline treatment, respectively, when compared with extraction performed with no pre-treatment. It also resulted in a reduced processing time (from 60 to 10 min). Extraction of R-phycoerythrin has been deemed effective by combining EAE and UAE in *Grateloupia turuturu* [26], due to a synergistic effect. In *Gelidium pusillum*, UAE followed by maceration and extraction in phosphate buffers allowed the recovery of 77% and 93% of R-PE and R-phycocyanin (R-PC), respectively [83]. Some examples are presented in Table 3.

Table 3. The conventional, enzyme-assisted and ultrasound-assisted cell disruption and protein extraction methods applied to different seaweeds.

Cell Disruption Method	Extraction Method	Reagents/Buffers	Conditions	Species	Initial Protein Content	Protein Recovery Yield	Protein Quantification Method	Ref.
Enzymatic hydrolysis	Polysaccharidase degradation and buffer treatment (sequential)	Phosphate buffer, commercial mixture of polysaccharidases containing cellulase, hemicellulase and β -glucanase, Tris-HCl.	Enzymatic pre-treatment 10 g freeze-dried algal powder; 200 mL of enzymatic medium, pH 6 (6 g of polysaccharidase powder in 200 mL of phosphate buffer 0.1 M, pH 6); 30 °C; 2 h. Buffer treatment After filtration through a nylon mesh, the residue was ground with a pestle and a mortar in 100 mL Tris-HCl (0.1 M, pH 7.5); 4 °C. Supernatant collected after centrifugation (10,000 $\times$ g, 20 min, 4 °C).	<i>Ulva rigida</i>	112.0 $\pm$ 5.8 g kg dw ⁻¹	18.5 $\pm$ 2.1% ^a	Kjeldahl method (N $\times$ 6.25)	[84]
				<i>Ulva rotundata</i>	100.1 $\pm$ 4.9 g kg dw ⁻¹	22.0 $\pm$ 1.5% ^a		
	Polysaccharidase degradation and alkaline treatment (sequential)	Deionized water, Cellulase and xylanase (Celluclast/Shearzyme), NaOH and NAC.	Enzymatic pre-treatment 1:30 (<i>w/v</i>) of dried milled seaweed to liquid suspension at pH 5 was pre-incubated (30 min, 40 °C). Enzyme:substrate (E:S) of 48.0 $\cdot$ 10 ³ units/100 g; reaction system incubated at 40 °C; 24 h. The supernatant was collected following centrifugation at 11,950 $\times$ g, room temperature. Alkaline treatment Pellet resuspended; weight:volume of 1:15; 0.12 M NaOH; 0.1% (<i>w/v</i>) NAC; stirred for 1 h; room temperature. Supernatant collected after centrifugation (11,950 $\times$ g, 20 min, room temperature).	<i>Palmaria palmata</i>	Not specified.	11.6 $\pm$ 0.08% dw ^b	Lowry method (modified)	[77]
High shear force	Aqueous treatment, Potter homogenization and alkaline treatment	Ultra-pure water, NaOH	Aqueous treatment 50 mg of freeze-dried sample; 4 mL ultra-pure water; 12 h; 4 °C. Potter homogenization for 5 min; 4 °C. Supernatant collected after centrifugation (15,000 $\times$ g, 20 min, 4 °C). Alkaline treatment Pellet resuspended in 1 mL of 0.1 N NaOH; shaking occasionally for 1 h; room temperature. Supernatant collected after centrifugation (15,000 $\times$ g, 20 min, room temperature).	<i>Porphyra acanthophora</i> var. <i>acanthophora</i>	16.5% dw	8.9 $\pm$ 0.7% dw ^b	Lowry method	[21]
				<i>Sargassum vulgare</i>	11.5% dw	6.9 $\pm$ 0.2% dw ^b		
				<i>Ulva fasciata</i>	12.8% dw	7.3 $\pm$ 0.8% dw ^b		
	Aqueous and alkaline treatment (sequential) with Ultra-Turrax homogenizer.	Deionized water, NaOH and NAC	Aqueous high shear force treatment Ultra-Turrax; 24,000 rpm; 1:20 of weight:volume of deionized water. Following shearing, stirred for 1 h; 4 °C. Supernatant collected after centrifugation (11,950 $\times$ g, 20 min, 4 °C). Alkaline treatment Pellet resuspended in 0.12 M NaOH; 0.1% (<i>w/v</i>) NAC; weight:volume of 1:15; stirred for 1 h; room temperature. Supernatant collected after centrifugation (11,950 $\times$ g, 20 min, room temperature). Supernatant collected after centrifugation and combined with that of the previous step.	<i>Palmaria palmata</i>	Not specified.	6.9 $\pm$ 0.1% dw ^b	Lowry method (modified)	[77]
	Buffer treatment with sonication	Tris-HCl	10 g of algal powder; 200 mL Tris-HCl (0.1 M pH 7.5); suspension submitted to ultrasound for 1 h (Ultrasonick 300 Ney, maximal power); 4 °C; stirred overnight. Supernatant collected after centrifugation (10,000 $\times$ g, 20 min, 4 °C).	<i>Ulva rigida</i>	112.0 $\pm$ 5.8 g kg dw ⁻¹	10.4 $\pm$ 0.8% ^a	Kjeldahl method (N $\times$ 6.25)	[84]
				<i>Ulva rotundata</i>	100.1 $\pm$ 4.9 g kg dw ⁻¹	16.1 $\pm$ 0.9% ^a		

Table 3. Cont.

Cell Disruption Method	Extraction Method	Reagents/Buffers	Conditions	Species	Initial Protein Content	Protein Recovery Yield	Protein Quantification Method	Ref.
	Sonication in aqueous conditions and ammonium sulfate-induced precipitation (sequential)	Ultra-pure water and ammonium sulfate	Sonication in aqueous conditions 10 g of freeze-dried and milled seaweed was suspended in 1 L of ultra-pure water; ultra-sonication for 1 h; left to stir overnight; 4 °C. Supernatant decanted after centrifugation (10,000× g, 1 h). Pellet suspended in 200 mL of ultra-pure water and subjected to a second extraction. Precipitation Supernatants were pooled and brought to 80% (w/v) ammonium sulfate saturation; stirred for 1 h; 4 °C; centrifuged (20,000× g, 1 h) to precipitate the protein fraction. Precipitates were dissolved and dialyzed using 3.5 kDa MWCO dialysis tubing against Milli-Q water; overnight; 4 °C.	<i>Ulva lactuca</i>	Not specified.	19.6 ± 0.6% ^a	Lowry method (modified)	[85]
	Aqueous treatment, alkaline solubilization and isoelectric precipitation (sequential)	Deionized water, NaOH and HCl	Aqueous treatment Dry-milled seaweed in distilled water in a 1:6 (w/v) ratio, based on the original wet weight of each species. Homogenization using Ultra-Turrax; 2 min; 18,000 rpm. Milling with beads; 2 min; 1/30 s. Homogenized sample was stirred for 1 h at 8 °C. Alkaline solubilization and isoelectric precipitation pH adjusted to 12; sample kept in ice. Supernatant collected after centrifugation (8000× g, 10 min). Adjusted to pH 2 and frozen overnight; −20 °C. After thawing and a second centrifugation (8000× g, 10 min), the pellet was collected and freeze dried.	<i>Saccharina latissima</i> <i>Porphyra umbilicalis</i>	Not specified. Not specified.	25.1 ± 0.9% ^a 22.6 ± 7.3% ^a	Lowry method (modified)	[85]
	Aqueous treatment	Deionized water	10 g of algal powder; 200 mL deionized water; 4 °C; stirred overnight. Supernatant collected after centrifugation (10,000× g, 20 min, 4 °C).	<i>Ulva rigida</i> <i>Ulva rotundata</i>	112.0 ± 5.8 g kg ^{dw} ^{−1} 100.1 ± 4.9 g kg ^{dw} ^{−1}	9.7 ± 0.6% ^a 14.0 ± 1.8% ^a	Kjeldahl method (N × 6.25)	[84]
Osmotic shock	Aqueous and alkaline treatment (sequential)	Deionized water and NaOH Deionized water, NaOH and NAC	Aqueous treatment 10 g of algal powder; 200 mL deionized water; 4 °C; stirred overnight. Alkaline treatment After centrifugation (10,000× g, 20 min, 4 °C), the pellet was treated with NaOH (0.1 M) and mercaptoethanol (0.5% v/v); stirred for 1 h; room temperature. Supernatant collected after centrifugation (10,000× g, 20 min, room temperature). Aqueous treatment Dried milled seaweed suspended in deionized water (1:20 (w/v); stirred for 16 h; 4 °C. Supernatant collected after centrifugation (11,950× g, 20 min, 4 °C). Alkaline treatment Pellet resuspended in 0.12 M NaOH; 0.1% (w/v) NAC; weight:volume of 1:15; stirred for 1 h; room temperature. Supernatant collected after centrifugation (11,950× g, 20 min, room temperature). Supernatant collected after centrifugation and combined with that of the previous step.	<i>Ulva rigida</i> <i>Ulva rotundata</i> <i>Palmaria palmata</i>	112.0 ± 5.8 g kg ^{dw} ^{−1} 100.1 ± 4.9 g kg ^{dw} ^{−1} Not specified.	26.8 ± 1.3% ^a 36.1 ± 1.4% ^a 6.7 ± 0.2% ^b	Kjeldahl method (N × 6.25) Lowry method (modified)	[84] [77]
	Buffer treatment	Tris-HCl	10 g of algal powder; 200 mL Tris-HCl (0.1 M pH 7.5); 4 °C; stirred overnight. Supernatant collected after centrifugation (10,000× g, 20 min, 4 °C).	<i>Ulva rigida</i> <i>Ulva rotundata</i>	112.0 ± 5.8 g kg ^{dw} ^{−1} 100.1 ± 4.9 g kg ^{dw} ^{−1}	9.4 ± 1.6% ^a 13.8 ± 1.2% ^a		
None	Buffer and alkaline treatment (sequential)	Tris-HCl and NaOH	Buffer treatment 10 g of algal powder; 200 mL Tris HCl (0.1 M pH 7.5); 4 °C; stirred overnight. Alkaline treatment After centrifugation (10,000× g, 20 min, 4 °C), the pellet was treated with NaOH (0.1 M) and mercaptoethanol (0.5% v/v); stirred for 1 h; room temperature. Supernatant collected after centrifugation (10,000× g, 20 min, room temperature).	<i>Ulva rigida</i> <i>Ulva rotundata</i>	112.0 ± 5.8 g kg ^{dw} ^{−1} 100.1 ± 4.9 g kg ^{dw} ^{−1}	17.5 ± 1.3% ^a 25.2 ± 1.9% ^a	Kjeldahl method (N × 6.25)	[84]
	Aqueous biphasic system	PEG/K ₂ CO ₃	PEG 1550 (10% w/v) and K ₂ CO ₃ (15% w/v). Algal powder (30 g/L) was mixed with PEG, suspended in Milli-Q water (15 min), extracted 15 min after the addition of the salt solution and centrifuged (4500× g, 25 min, 23 °C). Top phase-I was separated and stirred with the same volume of salt solution for 15 min and centrifuged (5000× g, 5 min, 23 °C). Anionic polysaccharides present a high affinity for the salt saturated aqueous phase and proteins show affinity for the polyethylene glycol phase.	<i>Ulva rigida</i> <i>Ulva rotundata</i>	112.0 ± 5.8 g kg ^{dw} ^{−1} 100.1 ± 4.9 g kg ^{dw} ^{−1}	19.1 ± 1.1% ^a 31.6 ± 2.1% ^a		

^a Total protein yield expressed as % of total protein (protein extracted/total protein × 100); ^b dw expressed as algal dry weight; NAC: N-acetyl-L-cysteine.

2.5. Novel Methods

The design of a biorefinery usually follows a fractionation approach, so as to maximize the number, quality and quantity of the extractable products and thus enhance the value of the bioresource. This is why novel extraction methods are being sought; e.g., in seaweed, which might yield a more complete valorization of the macroalgae by obtaining pure extracts in the first separation steps and, concomitantly, not causing harm to the subsequently extracted components.

2.5.1. Pulsed Electric Field Extraction

Pulsed Electric Field Extraction (PEFE) is used as a cell disruption method, particularly in microalgae. It involves the application of short, high-voltage electric current pulses to perforate a cell wall or membrane. Depending on the intensity, amplitude, duration, number and repetition frequency of the external electric pulses, reversible or irreversible pores (electroporation) are formed in the membranes. Irreversible pore formation is of particular importance for extraction of bioactive compounds from natural matrices [14]. Treatments with an electric field strength from 0.7 to 3 kV/cm, a specific energy of 1–20 kJ/kg, a couple of hundred of pulses and a total time duration lower than 1 s are usually used for natural products extraction [80]. When applied to *U. lactuca*, a higher protein content was observed, namely, 59 $\mu\text{g mL}^{-1}$ against the 23 $\mu\text{g mL}^{-1}$ obtained in the control [84]. When 50 pulses of 50 kV, applied at a 70.3 mm electrode gap, were used in the extraction of proteins from *Ulva* spp., a sevenfold increase in total protein content was obtained when compared to the use of osmotic shock [86]. The applied conditions can possibly limit the scale up of the method.

2.5.2. Microwave-Assisted Extraction

Microwave-Assisted Extraction (MAE) is another procedure used to enhance protein extraction from biological matrices. When microwave energy is transferred to the solution or suspension, the vibration/oscillation of the polar molecules occurs, causing inter- and intra-molecular friction. This effect combined with the movement and collision of a large number of charged particles leads to the heating of the matrices. Intracellular heating ultimately leads to pressurization effects that induce the breakdown of cell walls and membranes, in addition to electroporation effects [80]. Since MAE has been mostly used for analytical purposes (sample preparation), the results found in the literature focus on the reduction of extraction times rather than on its effects on parameters such as protein functionality [80].

This method can be carried out in open vessels (atmospheric pressure) or closed ones, under controlled pressure and temperature. In closed vessels, the solvent can be heated above its normal boiling point by manipulating the pressure, accelerating the mass transfer of the compounds from the natural matrix to the bulk solvent [87]. The higher the dielectric constant of the solvent and/or algal matrix, the greater the energy absorbed by the molecules and the faster the extraction temperature is reached [88,89], which makes polar solvents, such as water, the preferred choice.

2.5.3. Pressurized Liquid Extraction

Pressurized Liquid Extraction (PLE), or Accelerated Solvent Extraction (ASE), combines temperatures that range from 50 to 200 °C and pressure ranging from 35 to 200 bar. These parameters are set for values lower than the solvent critical temperature and pressure, keeping it in its liquid state [20]. Both temperature and pressure increase the mass transfer rate. High pressures cause the solvent to reach temperatures higher than its normal boiling point and these higher temperatures enhance the solubility of the extractable components and reduce the viscosity and surface tension of the solvent [80]. Water is the most widely used solvent but other solvents, such as propane and dimethyl ether (DME), can also be

used. Since DME is partially miscible with water, it allows the simultaneous extraction of non-polar target metabolites and the removal of water from wet matrices [90–92].

2.5.4. Supercritical Fluid Extraction

Supercritical Fluid Extraction (SFE) is an alternative extraction technique that produces extracts with very low contents of polar impurities [93]. It is a green technology, since a concentration step can most often be skipped after the extraction process [94]. A fluid becomes supercritical if subjected to temperature and pressure conditions above its critical point values (P_c ; T_c). Experimental studies using SFE are usually limited to the region of $P_c < P \leq 6P_c$ and $T_c < T \leq 1.4T_c$ [95]. Under supercritical conditions, some of the properties of the fluid become indistinguishable from those of its gaseous state, with a density similar to that of a liquid, but with diffusivity and viscosity matching those of a gas. This makes supercritical fluids capable of a faster and deeper penetration into the solid particles of the matrix to be processed [93]. The fluid must be chosen carefully, especially when dealing with thermolabile compounds. Carbon dioxide, with T_c and P_c values of 31.1 °C and 73.9 bar, is by far the most used supercritical solvent [96]. In addition to being ideal for the extraction of thermolabile compounds, it has low viscosity, low surface tension, high diffusivity, is non-toxic, non-flammable, widely available and chemically inert under the conditions relevant for the extraction process. The fact that it is gaseous at normal pressure and temperature eliminates the need for a solvent evaporation step after extraction [96]. The greatest limitation of supercritical CO₂ is that it is not suitable for extraction of polar compounds [97].

The addition of an organic modifier, such as ethanol or methanol, can greatly improve the extraction efficiency with supercritical CO₂ [96]. Other possible supercritical solvents, such as water, methanol, ethanol, acetone, chloroform, ethyl acetate and toluene, are usually avoided when extracting bioactive compounds, since their T_c value is above 200 °C [80].

The SFE technology has been used for processing seaweeds and other plant-based materials mainly to extract non-polar and small-size molecular components, such as pigments (chlorophylls, carotenoids) and some lipids (e.g., sterols) that are valuable as food and pharmaceutical ingredients. Proteins, polysaccharides, long-chain fatty acids and phlorotannins have also been recovered from seaweeds under PLE using subcritical solvents, and/or subcritical water and organic modifiers (SWE) [98]. Therefore, SFE may be a first processing step in seaweed biorefinery strategy [85] wherein the protein extraction step, from the first-extracted biomass, could be done either by PLE, SWE or conventional methods, after optimizing the operating conditions to increase the selectivity, as recommended for biorefineries of other plant-based resources [98].

2.5.5. Switchable or Smart Solvents

Switchable solvents are a new class of smart extraction solvents that can switch from a non-ionic form to an ionic liquid through contact with CO₂ gas bubbles. Exposure to N₂ allows them to return to their non-ionic form [14]. Ionic liquids (ILs) are composed of large asymmetric organic cations (imidazolium, pyrrolidinium, pyridinium, ammonium or phosphonium) and different inorganic or organic anions, such as BF₄[−], PF₆[−], Cl[−] and Br[−] [80]. They are very versatile, since their polarity, hydrophobicity, viscosity and other properties highly depend on the cationic or anionic constituents selected, hence being referred to as “designer solvents”. They have low melting points (below 100 °C) and their non-flammable and non-volatile nature makes them an attractive choice for the development of safer processes [80].

The basis of the extraction mechanism with ILs lies in their interaction with macroalgal cell walls. As mentioned, cellulose is a core component of algae cell walls, although its proportion varies between species. The use of a solvent capable of disrupting the intermolecular H-bond interactions leads to cellulose dissolution [99]. Indeed, several ILs have been found to dissolve large amounts of cellulose [100]. These interactions are relevant because they ultimately lead to the complete or partial disruption of cell walls [101].

3. Protein Enrichment Methods

Using seaweed as a protein resource requires the processing of biomass to deliver a concentrated form of high-quality protein. The protein concentration from plant materials has traditionally been achieved directly by extracting and isolating the protein, or indirectly by extracting the non-protein components. In contrast with other plant materials, isolating and concentrating proteins from seaweed is a relatively unexplored domain and most protocols tend to focus solely on the initial extraction methods [102]. Purification of the extracted proteins represents a challenge, especially for novel proteins because of their unknown physicochemical properties. The selection of the method also depends on the final application of the product and the scale of production. The extraction method used also influences the purification method chosen. For example, reducing agents, such as N-acetyl-L-cysteine, which are used to improve cell wall-associated protein extraction, might have to be avoided, depending on the applications of the extracted proteins.

Single or combined enrichment methods can be employed. These methods include precipitation, membrane technologies and chromatography [14]. A combination of membrane technologies could be used to isolate seaweed proteins using the same principles used to obtain protein isolates in the dairy industry. Microfiltration (MF) could be used to remove the cell wall components, ultrafiltration (UF) could be used to isolate proteins with a molecular weight between 1 and 200 kDa, nanofiltration (NF) could be used to remove monovalent salts and reverse osmosis (RO) to reduce the final volume [103]. Ultrafiltration has been applied after supercritical CO₂ and ultrasonic-assisted extraction to isolate polysaccharides from *Sargassum pallidum* [25] and after hot water extraction in *Ulva fasciata* [104]. Most protocols reported in the literature often describe methods for the purification and isolation of specific proteins, namely, R-PE. Some examples are presented in Table 4.

Table 4. Examples of proteins from algae species and the methods tested for their purification.

Aim	Extraction Method	Enrichment Method	Conditions	Species	Results	Ref.
Concentrate R-PE; pre-purify by eliminating proteins other than R-PE and polysaccharides.	Centrifugation of algal powder after suspension in distilled water.	Ultrafiltration	PES membrane; MWCO of 25–30 kDa; surface area of 0.033 m ² ; 20 °C; 4 bar; volumetric concentration factor of 5.	<i>Grateloupia turuturu</i>	100% of R-PE recovered; 32.9% of other proteins and 64.6% of sugars passed through the membrane.	[105]
Concentrate phycobiliproteins (R-PE and allophycocyanin).	Mixing of algal powder in 50 mM citrate buffer (pH 6) for 24 h and centrifugation of the suspension.	Microfiltration; Ultrafiltration and SEC	Microfiltration and Ultrafiltration membranes of RC, 0.45 µm, and of PES, 50 kDa of MWCO. SEC Mobile phase: Phosphate buffer (pH 7.2, 50 mM sodium phosphate and 150 mM NaCl); room temperature. Stationary phase with 34 µm average particle size.	<i>Furcellaria lumbricalis</i>	60–75% of R-PE and allophycocyanin were recovered values dependent on the detector, (fluorescence or photodiode array).	[106]

Table 4. Cont.

Aim	Extraction Method	Enrichment Method	Conditions	Species	Results	Ref.
Isolate R-PE.	Mixing of fresh thallus in phosphate buffer (0.02 mM; pH 7.2); pulverization of the mixture.	Isoelectric Precipitation and AEX	Filtration (gauze); repeated freezing and thawing; centrifugation; supernatant precipitation with 35% saturated ammonium sulfate; supernatant precipitation with 55% saturated ammonium sulfate; centrifugation; dialysis against 50 mM phosphate buffer (pH 7.2). Q-Sepharose column; flow rate of 2.0 mL/min; elution with 50 mM phosphate buffer (pH 7.2) with an increasing gradient of NaCl (0 to 200 mM); elution of the active fraction occurs at a concentration of NaCl of 200 mM.	<i>Portieria hornemannii</i>	R-PE recovery of 64.8% with purity of 5.2%.	[107]
Isolate R-PE.	Dried algal hydration with deionized water overnight; slurry filtration through gauze.	Supernatant precipitation with ammonium sulfate (final concentration of 0.5 M). EBA and AEX.	EBA —Streamline™ column; supernatant injected with the crude extracts; eluates pooled and dialyzed against distilled water overnight at 4 °C. AEX —DEAE-Sepharose column; flow rate of 2.5 mL/min; sodium acetate (NaAc) buffer (4 mM; pH 4.5) eliminates phycocyanin contaminants; 1 mM NaAc buffer (pH 4.5) and phosphate buffer (50 mM; pH 6.8) eliminates other contaminant proteins; elution with phosphate buffer (30 mM; pH 6.8) using an increasing gradient of NaCl from 0 to 200 mM.	<i>Gracilaria lemaneiformis</i>	R-PE recovery of 21% with purity ratio >3.2.	[108]

AEX—anion-exchange chromatography; EBA—expanded bed adsorption; MWCO—molecular weight cut-off; PES—polyethersulfone; RC—regenerated cellulose; SEC—size-exclusion chromatography.

4. Protein Characterization Methods

Characterization and/or identification of isolated proteins is usually carried out by direct comparison with standard molecules and/or data collected from the available literature. This approach is successful until unknown compounds are brought into consideration for which standards are not available [14]. Exploratory methods thus have to be used.

4.1. SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is used to identify the molecular weight of dominant protein subunits bands (Table 5). This method has been used to identify the protein profiles of different seaweeds, such as *Ulva* spp. [28], *Gracilaria changii* [109], *Caulerpa lentillifera*, *Caulerpa racemosa* and *Kappaphycus* spp., amongst others [110].

Table 5. Protein isolation and characterization using SDS-PAGE methods in different seaweed species.

Species	Identified Proteins	Molecular weight	Method	Ref.
<i>Eisenia bicyclis</i>	EHEP (<i>Eisenia</i> hydrolysis enhancing protein)	25 kDa	SDS-PAGE and 2D-PAGE	[111]
<i>Furcellaria lumbricalis</i>	R-PE	~25 kDa	SDS-PAGE using 4–15% Mini-Protean® TGX Stain Free Precast Gel.	[106]
<i>Himantalia elongata</i>	5 proteins	71.6, 53.7, 43.3, 36.4, 27.1 kDa	Tris-Tricine-SDS-PAGE using 10–20% Mini-Protean® Tris-Tricine Precast Gel.	[112]
<i>Laminaria japonica</i>	LJGP (<i>Laminaria japonica</i> novel glycoprotein)	~10 kDa	SDS-PAGE on 15% gels; periodic acid-Schiff staining for glycoprotein bands.	[113]
<i>Palmaria palmata</i>	One prominent area of staining (suspected of being subunits of phycoerythrin or other phycobiliproteins).	~20 kDa	SDS-PAGE using a Mini-Protean® II electrophoresis system with 4 g/100 mL acrylamide stacking gel and 12.5 g/100 mL acrylamide resolving gel.	[77]
<i>Pyropia yezoensis</i>	2 proteins: PYP1 and PYP2 (<i>Pyropia yezoensis</i> porphyrin 1 and 2).	PYP1: 10 kDa, SDS-resistant dimer; PYP2: 10 kDa.	SDS-PAGE using a 18% acrylamide gel.	[114]

4.2. Chromatography

As mentioned in Section 3, chromatographic methods are widely used for separation and purification of seaweed proteins and they can be used also for their characterization. High-Performance Liquid Chromatography (HPLC) is often used coupled with other chromatographic methods, such as Size-Exclusion Chromatography (HPSEC) [106]. Amino acid profiles after protein digestion can be analyzed with gaseous or liquid chromatography (GC or LC) with a derivatization step, which includes treatment with ortho-phthalaldehyde (OPA) or fluorenylmethoxy chloroformate (FMOC) [115,116]. However, assessment of the amino acid profile without derivatization by using anion exchange chromatography (HPAEC-PAD) has been reported [117]. Examples of chromatographic methods used for seaweed protein isolation and characterization are shown in Table 6.

Table 6. Protein isolation and characterization using chromatographic methods in different seaweed species.

Species	Identified Proteins	Molecular Weight	Method	Ref.
<i>Ascophyllum nodosum</i>	Protein profile	From 2.6 to 3.8 kDa	HPLC and SEC (HPSEC); macroporous HPLC column with particle size of 4–6 µm and pore size of 150–300 Å;	[106]
<i>Saccharina latissima</i>	Trypanothione reductase and ATP synthase subunit beta (chloroplastic); actin-1; elongation factor Tu; glyceraldehyde-3-phosphate dehydrogenase.	51, 41, 40, 39 kDa (respectively)	HPSEC and SDS-PAGE; 4–20% Precast Mini-Protean® linear gel; two serially connected columns—1st with 5 µm particle size, 150 Å pore size, 2nd with 5 µm particle size and 300 Å pore size.	[118]

4.3. Spectrometry

Fourier Transform Infrared (FTIR) spectroscopy is used to obtain information about the structural composition of proteins, especially when it comes to their secondary structural conformation [27]. Mass spectrometry (MS) can be an accurate protein identification tool and electrospray ionization (ESI) and matrix-assisted laser desorption ionizations/time-

of-flight (MALDI-TOF) can also be important tools [27]. Examples of their application to seaweed proteins are shown in Table 7.

Table 7. Protein characterization using spectrometry methods in different seaweed species.

Species	Structural Features Searched and Respective Bands, or Identified Proteins/Peptides	Method	Ref.
<i>Codium fragile</i>	Codiase	Fibrin(ogen)olytic activity of codiase was examined by FTIR spectroscopy and the molecular weight of codiase was determined by MALDI-TOF mass spectrometry in linear mode.	[40]
<i>Kappaphycus alvarezzi</i>	704 cm ⁻¹ : N—H bending. 616 cm ⁻¹ : phosphate group.	The lyophilized protein concentrate was pelletized with potassium bromide (1/100 ratio <i>w/w</i>) and the spectral analysis was carried out using FTIR.	[119]
<i>Macrocystis pyrifera</i> and <i>Chondracanthus chамissoi</i>	3281 cm ⁻¹ and 3274 cm ⁻¹ : N—H vibrations. 1637 cm ⁻¹ and 1544 cm ⁻¹ : C=O vibrations. 1220 cm ⁻¹ and 1243 cm ⁻¹ : S=O vibrations.	FTIR of dried seaweed and protein extracts of seaweeds.	[75]
<i>Palmaria palmata</i> and <i>Soliera chordalis</i>	Bioactive peptides	Peptide samples were separated by online reversed-phase (RP) nanoscale capillary liquid chromatography (nanoLC) and analyzed by electrospray mass spectrometry (ES MS/MS).	[49]
<i>Palmaria palmata</i> , <i>Palmaria umbilicalis</i> , <i>Ulva rigida</i> , <i>Ulva pinnatifida</i> and <i>Laminaria zchroleuca</i>	Mono-iodotyrosine and diiodotyrosine	Reverse phase high performance liquid chromatography (RP-HPLC) with inductively coupled plasma mass spectrometry (ICP-MS).	[120]

5. Conclusions

Given the current interest in sustainable protein sources, macroalgae have been identified as promising alternatives due to their protein quality and bioactivities. Seaweed proteins and seaweed-derived proteins are still poorly explored, and the development of appropriate and feasible extraction and purification processes is of growing interest in order to incorporate them in pharmaceutical, nutraceutical, cosmeceutical or food and feed applications. The major identified challenges lie on improving the protein yield and on the disruption of the polysaccharide-rich cell wall. Cell disruption techniques play indeed an essential role in the successful extraction and enrichment of algal protein at larger scales. Additionally, the protein contents and quality highly depend on parameters such as the species, harvesting season, location and growing conditions.

Conventional protein extraction methods, such as enzymatic hydrolysis and various physical processes, are laborious, time-consuming and costly, and may require the use of solvents. Novel methods, such as PEF, MAE, PLE and SFE, have been predominantly used in the extraction of compounds other than proteins, and are still poorly explored in algae. Table 8 briefly compares relevant aspects of these conventional and novel extraction methods.

Table 8. Comparison of the relevant aspects of protein extraction methods.

Aim/Method	Main Advantages	Main Drawbacks	Ease of Scale-Up **
<i>Cell wall disruption</i>			
Osmotic shock	Low energy; selectivity	Cost of chemicals; diluted product	+++
Mechanical shearing	No chemicals; high yield	Energy cost; heat damage *	+++
Enzyme treatment (EAE)	Low energy; selectivity	Cost of enzymes; product contaminated with enzyme	++
Ultrasonication (UAE)	No chemicals; high yield	Energy cost; heat damage *; equipment cost	++
Pulsed electric field (PEFE)	No chemicals; high yield	Energy cost; heat damage *; equipment cost	+
Microwave (MAE)	No chemicals; high yield	Energy cost; heat damage *; equipment cost	+
<i>Protein extraction</i>			
Aqueous solubilization (water, alkali, buffer)	Mild conditions; bioactivity preserved	Limited recovery yield; diluted product	+++
Aqueous biphasic systems	Mild conditions; bioactivity preserved; achievable high recovery yield and selectivity;	Cost of chemicals; improvements depend on cost and environmental impact	++
Pressurized liquid extraction (PLE)/Accelerated solvent extraction (ASE)	Higher recovery yield	Energy cost; heat damage *; equipment cost; possible product contamination by co-solvents	++
Enzymatic protein hydrolysis (EAE)	Higher recovery yield; tailored protein modification; possible gain of peptide bioactivities	Cost of enzymes; loss of protein bioactivity/function	++
<i>Protein enrichment</i>			
Precipitation	High recovery yield; mild conditions	Cost of chemicals; low selectivity	+++
Membrane separation (MF, UF, NF, RO)	High recovery yield; no chemicals; mild conditions	Cost of membranes; low selectivity	+++
Adsorption/Chromatography	High selectivity; high recovery yield	Cost of adsorber media, instrumentation and buffer chemicals	++
Supercritical fluid extraction (SFE)	High selectivity for low polarity impurities	Energy cost; equipment cost; possible product contamination by co-solvents	+

* Need of thermal control. ** From + (minimum) to +++ (maximum).

To achieve progress in this protein extraction area, future work addressing the applicability of these novel methods is necessary. The same applies to membrane technologies, which also show great promise to pre-purify/concentrate or even isolate (in membrane chromatography) compounds, but are still fully underexploited regarding the protein extraction from macroalgae.

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References

1. IEA Bioenergy Task 42 Biorefining. Available online: <https://task42.ieabioenergy.com/> (accessed on 20 August 2021).
2. Stengel, D.B.; Connan, S. Marine Algae: A Source of Biomass for Biotechnological Applications. In *Natural Products from Marine Algae*; Humana Press: New York, NY, USA, 2015; Volume 1308, pp. 1–37. [CrossRef]
3. Chojnacka, K.; Wiczorek, P.; Schroeder, G.; Michalak, I. *Algae Biomass: Characteristics and Applications*, 8th ed.; Springer International Publishing: Cham, Switzerland, 2018. [CrossRef]
4. Bold, H.; Wynne, M. *Introduction to the Algae: Structure and Reproduction*; Prentice-Hall: Englewood Cliffs, NJ, USA, 1978.
5. Lobban, C.; Wynne, M. *The Biology of Seaweeds*; University of California Press: Berkeley, CA, USA, 1981.
6. Van den Hoek, C.; Mann, D.; Jahns, H. *Algae: An Introduction to Phycology*; Cambridge University Press: Berkeley, CA, USA, 1995. [CrossRef]
7. Reed, R.; Lobban, C.; Harrison, P.; Duncan, M. The Physiological Ecology of Seaweeds. *J. Ecol.* **1986**, *74*, 611. [CrossRef]
8. Santelices, B. Production Ecology of *Gelidium*. *Hydrobiologia* **1991**, *221*, 31–44. [CrossRef]
9. Gao, K.; McKinley, K.R. Use of Macroalgae for Marine Biomass Production and CO₂ Remediation: A Review. *J. Appl. Phycol.* **1994**, *6*, 45–60. [CrossRef]
10. Percival, E.; McDowell, R. Algal Polysaccharides. In *Methods in Plant Biochemistry*; Academic Press: Cambridge, MA, USA, 1990; Volume 2, pp. 523–547. [CrossRef]
11. Cesário, M.T.; da Fonseca, M.M.R.; Marques, M.M.; de Almeida, M.C.M.D. Marine Algal Carbohydrates as Carbon Sources for the Production of Biochemicals and Biomaterials. *Biotechnol. Adv.* **2018**, *36*, 798–817. [CrossRef] [PubMed]
12. Lahaye, M.; Robic, A. Structure and Function Properties of Ulvan, a Polysaccharide from Green Seaweeds. *Biomacromolecules* **2007**, *8*, 1765–1774. [CrossRef] [PubMed]
13. McHugh, D. *A Guide to the Seaweed Industry*; Food and Agriculture Organization of the United States: Rome, Italy, 2003.
14. Pliego-Cortés, H.; Wijesekara, I.; Lang, M.; Bourgougnon, N.; Bedoux, G. Current Knowledge and Challenges in Extraction, Characterization and Bioactivity of Seaweed Protein and Seaweed-Derived Proteins. *Adv. Bot. Res.* **2020**, *95*, 289–326. [CrossRef]
15. Fleurence, J. Seaweed Proteins. In *Proteins in Food Processing*; Elsevier Ltd.: Amsterdam, The Netherlands, 2004; pp. 197–213. [CrossRef]
16. Sánchez-Machado, D.; López-Cervantes, J.; López-Hernández, J.; Paseiro-Losada, P. Fatty Acids, Total Lipid, Protein and Ash Contents of Processed Edible Seaweeds. *Food Chem.* **2004**, *85*, 439–444. [CrossRef]
17. Biris-Dorhoi, E.S.; Michiu, D.; Pop, C.; Rotar, A.; Tofana, M.; Pop, O.; Socaci, S.; Farcas, A. Macroalgae—A Sustainable Source of Chemical Compounds with Biological Activities. *Nutrients* **2020**, *12*, 3085. [CrossRef] [PubMed]
18. Pimentel, F.; Alves, R.; Harnedy, P.; Fitzgerald, R.; Oliveira, M. Macroalgal-Derived Protein Hydrolysates and Bioactive Peptides: Enzymatic Release and Potential Health Enhancing Properties. *Trends Food Sci. Technol.* **2019**, *93*, 106–124. [CrossRef]
19. Marsham, S.; Scott, G.; Tobin, M. Comparison of Nutritive Chemistry of a Range of Temperate Seaweeds. *Food Chem.* **2007**, *4*, 1331–1336. [CrossRef]
20. Day, L. Proteins from Land Plants—Potential Resources for Human Nutrition and Food Security. *Trends Food Sci. Technol.* **2013**, *32*, 25–42. [CrossRef]
21. Barbarino, E.; Lourenço, S. An Evaluation of Methods for Extraction and Quantification of Protein from Marine Macro- and Microalgae. *J. Appl. Phycol.* **2005**, *17*, 447–460. [CrossRef]
22. Machado, M.; Machado, S.; Pimentel, F.; Freitas, V.; Alves, R.; Oliveira, M. Amino Acid Profile and Protein Quality Assessment of Macroalgae Produced in an Integrated Multi-Trophic Aquaculture System. *Foods* **2020**, *9*, 1382. [CrossRef]
23. Cian, R.; Drago, S.; de Medina, F.; Martínez-Augustín, O. Proteins and Carbohydrates from Red Seaweeds: Evidence for Beneficial Effects on Gut Function and Microbiota. *Mar. Drugs* **2015**, *13*, 5358–5383. [CrossRef]
24. Mittal, R.; Sharma, R.; Raghavarao, K. Aqueous Two-Phase Extraction of R-Phycoerythrin from Marine Macro-Algae, *Gelidium pusillum*. *Bioresour. Technol.* **2019**, *280*, 277–286. [CrossRef]
25. Denis, C.; Massé, A.; Fleurence, J.; Jaouen, P. Concentration and Pre-Purification with Ultrafiltration of a R-Phycoerythrin Solution Extracted from Macro-Algae *Grateloupia turuturu*: Process Definition and up-Scaling. *Sep. Purif. Technol.* **2009**, *69*, 37–42. [CrossRef]
26. Le Guillard, C.; Dumay, J.; Donnay-Moreno, C.; Bruzac, S. Ultrasound-Assisted Extraction of R-Phycoerythrin from *Grateloupia turuturu* with and without Enzyme Addition. *Algal Res.* **2015**, *12*, 522–528. [CrossRef]
27. Pliego-Cortés, H.; Caamal-Fuentes, E.; Montero-Muñoz, J.; Freile-Pelegrín, Y.; Robledo, D. Growth, Biochemical and Antioxidant Content of *Rhodymenia pseudopalmeta* (Rhodymeniales, Rhodophyta) Cultivated under Salinity and Irradiance Treatments. *J. Appl. Phycol.* **2017**, *29*, 2595–2603. [CrossRef]
28. Wijesekara, I.; Lang, M.; Marty, C.; Gemin, M. Different Extraction Procedures and Analysis of Protein from *Ulva* sp. in Brittany, France. *J. Appl. Phycol.* **2017**, *29*, 2503–2511. [CrossRef]

29. Abdel-fattah, A.; Sary, H.H. Glycoproteins from *Ulva lactuca*. *Phytochemistry* **1987**, *26*, 1447–1448. [\[CrossRef\]](#)
30. Yoshiie, T.; Maeda, M.; Kimura, M.; Hama, Y.; Uchida, M.; Kimura, Y. Structural Features of N-Glycans of Seaweed Glycoproteins: Predominant Occurrence of High-Mannose Type N-Glycans in Marine Plants. *Biosci. Biotechnol. Biochem.* **2012**, *76*, 1996–1998. [\[CrossRef\]](#)
31. Kimura, N.; Uchida, M.; Nishimura, S.; Yamaguchi, H. Promotion of Polypeptide Folding by Interactions with Asn-Glycans. *J. Biochem.* **1998**, *124*, 857–862. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Mori, T.; O'Keefe, B.; Sowder, R.; Bringans, S.; Gardella, R.; Berg, S.; Cochran, P.; Turpin, J.; Buckheit, R.; McMahon, J.; et al. Isolation and Characterization of Griffithsin, a Novel HIV-Inactivating Protein, from the Red Alga *Griffithsia* sp. *J. Biol. Chem.* **2005**, *280*, 9345–9353. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Chaves, R.; Silva, S.; Neto, L.; Carneiro, R.; Silva, A.; Sampaio, A.; Sousa, B.; Cabral, M.; Videira, P.; Teixeira, E.; et al. Structural Characterization of Two Isolectins from the Marine Red Alga *Solieria filiformis* (Kützinger) P.W. Gabrielson and Their Anticancer Effect on MCF-7 Breast Cancer Cells. *Int. J. Biol. Macromol.* **2018**, *107*, 1320–1329. [\[CrossRef\]](#)
34. Mu, J.; Hirayama, M.; Sato, Y.; Morimoto, K.; Hori, K. A Novel High-Mannose Specific Lectin from the Green Alga *Halimeda renshu* Exhibits a Potent Anti-Influenza Virus Activity through High-Affinity Binding to the Viral Hemagglutinin. *Mar. Drugs* **2017**, *15*, 255. [\[CrossRef\]](#)
35. Fernández, P.; Ciancia, M.; Estevez, J. Cell Wall Variability in the Green Seaweed *Codium vermilara* (Bryopsidales Chlorophyta) from the Argentine Coast. *J. Phycol.* **2011**, *47*, 802–810. [\[CrossRef\]](#) [\[PubMed\]](#)
36. Estevez, J.; Fernández, P.; Kasulin, L.; Dupree, P.; Ciancia, M. Chemical and in Situ Characterization of Macromolecular Components of the Cell Walls from the Green Seaweed *Codium fragile*. *Glycobiology* **2009**, *19*, 212–228. [\[CrossRef\]](#)
37. Yang, D.; Wang, J.; Bao, Y.; An, L. Purification and Characterization of *Ulva pertusa* Kjellm Alkaline Phosphatase. *Prep. Biochem. Biotechnol.* **2003**, *33*, 113–123. [\[CrossRef\]](#)
38. Ünlü, E.; Ünüvar, Ö.; Aydın, M. Identification of Alternative Oxidase Encoding Genes in *Caulerpa cylindracea* by de Novo RNA-Seq Assembly Analysis. *Mar. Genom.* **2019**, *46*, 41–48. [\[CrossRef\]](#)
39. Matsubara, K.; Hori, K.; Matsuura, Y.; Miyazawa, K. A Fibrinolytic Enzyme from a Marine Green Alga, *Codium latum*. *Phytochemistry* **1999**, *52*, 993–999. [\[CrossRef\]](#)
40. Choi, J.; Sapkota, K.; Park, S.; Kim, S.; Kim, S. Thrombolytic, Anticoagulant and Antiplatelet Activities of Codinase, a Bi-Functional Fibrinolytic Enzyme from *Codium fragile*. *Biochimie* **2013**, *95*, 1266–1277. [\[CrossRef\]](#)
41. Tee, M.; Yong, Y.; Rodrigues, K.; Thau, W.; Yong, L. Growth Rate Analysis and Protein Identification of *Kappaphycus alvarezii* (Rhodophyta, Gigartinales) under pH Induced Stress Culture. *Aquac. Rep.* **2015**, *2*, 112–116. [\[CrossRef\]](#)
42. Harnedy, P.; Fitzgerald, R. Bioactive Proteins, Peptides, and Amino Acids from Macroalgae. *J. Phycol.* **2011**, *47*, 218–232. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Paiva, L.; Lima, E.; Patarra, R.; Neto, A.; Baptista, J. Edible Azorean Macroalgae as Source of Rich Nutrients with Impact on Human Health. *Food Chem.* **2014**, *164*, 128–135. [\[CrossRef\]](#)
44. Cermeño, M.; Kleekayai, T.; Amigo-Benavent, M.; Harnedy-Rothwell, P.; Fitzgerald, R.J. Current Knowledge on the Extraction, Purification, Identification, and Validation of Bioactive Peptides from Seaweed. *Electrophoresis* **2020**, *41*, 1694–1717. [\[CrossRef\]](#)
45. García-Cañas, V.; Simó, C.; Herrero, M.; Ibáñez, E.; Cifuentes, A. Present and Future Challenges in Food Analysis: Foodomics. *Anal. Chem.* **2012**, *84*, 10150–10159. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Bleakley, S.; Hayes, M. Algal Proteins: Extraction, Application, and Challenges Concerning Production. *Foods* **2017**, *6*, 33. [\[CrossRef\]](#) [\[PubMed\]](#)
47. Pangestuti, R.; Kim, S. Seaweed Proteins, Peptides, and Amino Acids. In *Seaweed Sustainability: Food and Non-Food Applications*; Elsevier Inc.: Amsterdam, The Netherlands, 2015; pp. 125–140. [\[CrossRef\]](#)
48. Guedes, A.; Amaro, H.; Sousa-Pinto, I.; Malcata, F. Applications of Spent Biomass. In *Biofuels from Algae*; Elsevier Inc.: Amsterdam, The Netherlands, 2013; pp. 205–233. [\[CrossRef\]](#)
49. Bondu, S.; Bonnet, C.; Gaubert, J.; Deslandes, É.; Turgeon, S.; Beaulieu, L. Bioassay-Guided Fractionation Approach for Determination of Protein Precursors of Proteolytic Bioactive Metabolites from Macroalgae. *J. Appl. Phycol.* **2015**, *27*, 2059–2074. [\[CrossRef\]](#)
50. Øverland, M.; Mydland, L.; Skrede, A. Marine Macroalgae as Sources of Protein and Bioactive Compounds in Feed for Monogastric Animals. *J. Sci. Food Agric.* **2019**, *99*, 13–24. [\[CrossRef\]](#) [\[PubMed\]](#)
51. Garcia-Vaquero, M.; Hayes, M. Red and Green Macroalgae for Fish and Animal Feed and Human Functional Food Development. *Food Rev. Int.* **2015**, *32*, 15–45. [\[CrossRef\]](#)
52. Foegeding, E.; Davis, J. Food Protein Functionality: A Comprehensive Approach. *Food Hydrocoll.* **2011**, *25*, 1853–1864. [\[CrossRef\]](#)
53. Bouwhuis, M.; Sweeney, T.; Mukhopadhyay, A.; McDonnell, M.; O'Doherty, J. Maternal Laminarin Supplementation Decreases *Salmonella typhimurium* Shedding and Improves Intestinal Health in Piglets Following an Experimental Challenge with *S. typhimurium* Post-Weaning. *Anim. Feed. Sci. Technol.* **2017**, *223*, 156–168. [\[CrossRef\]](#)
54. Sweeney, T.; Meredith, H.; Ryan, M.; Gath, V.; Thornton, K.; O'Doherty, J. Effects of *Ascomyces nodosum* Supplementation on *Campylobacter jejuni* Colonisation, Performance and Gut Health Following an Experimental Challenge in 10 Day Old Chicks. *Innov. Food Sci. Emerg. Technol.* **2016**, *37*, 247–252. [\[CrossRef\]](#)
55. Vatsos, I.; Rebours, C. Seaweed Extracts as Antimicrobial Agents in Aquaculture. *J. Appl. Phycol.* **2015**, *27*, 2017–2035. [\[CrossRef\]](#)

56. Sekar, S.; Chandramohan, M. Phycobiliproteins as a Commodity: Trends in Applied Research, Patents and Commercialization. *J. Appl. Phycol.* **2008**, *20*, 113–136. [\[CrossRef\]](#)
57. Smit, A. Medicinal and Pharmaceutical Uses of Seaweed Natural Products: A Review. *J. Appl. Phycol.* **2004**, *16*, 245–262. [\[CrossRef\]](#)
58. Murray, B.; Fitzgerald, R. Angiotensin Converting Enzyme Inhibitory Peptides Derived from Food Proteins: Biochemistry, Bioactivity and Production. *Curr. Pharm. Des.* **2007**, *13*, 773–791. [\[CrossRef\]](#)
59. Meisel, H. Multifunctional Peptides Encrypted in Milk Proteins. *BioFactors* **2004**, *21*, 55–61. [\[CrossRef\]](#)
60. Xu, W.; Liao, X.; Xu, S.; Diao, J.; Du, B.; Zhou, X.; Pan, S. Isolation, Structure Determination, and Synthesis of Galaxamide, a Rare Cytotoxic Cyclic Pentapeptide from a Marine Algae *Galaxaura filamentosa*. *Org. Lett.* **2008**, *10*, 4569–4572. [\[CrossRef\]](#)
61. Harnedy, P.; Fitzgerald, R. In Vitro Assessment of the Cardioprotective, Anti-Diabetic and Antioxidant Potential of *Palmaria palmata* Protein Hydrolysates. *J. Appl. Phycol.* **2013**, *25*, 1793–1803. [\[CrossRef\]](#)
62. Furuta, T.; Miyabe, Y.; Yasui, H.; Kinoshita, Y.; Kishimura, H. Angiotensin I Converting Enzyme Inhibitory Peptides Derived from Phycobiliproteins of Dulse *Palmaria palmata*. *Mar. Drugs* **2016**, *14*, 32. [\[CrossRef\]](#)
63. Cha, S. Screening of Extracts from Marine Green and Brown Algae in Jeju for Potential Marine Angiotensin-I Converting Enzyme (ACE) Inhibitory Activity. *J. Korean Soc. Food Sci. Nutr.* **2006**, *35*, 307–314. [\[CrossRef\]](#)
64. Saito, M. Antihypertensive Effect of Nori-Peptides Derived from Red Alga *Porphyra yezoensis* in Hypertensive Patients. *Am. J. Hypertens.* **2002**, *15*, A210. [\[CrossRef\]](#)
65. Sato, M.; Oba, T.; Yamaguchi, T.; Nakano, T.; Kahara, T.; Funayama, K.; Kobayashi, A.; Nakano, T. Antihypertensive Effects of Hydrolysates of Wakame (*Undaria pinnatifida*) and Their Angiotensin-I-Converting Enzyme Inhibitory Activity. *Ann. Nutr. Metab.* **2002**, *46*, 259–267. [\[CrossRef\]](#)
66. Maehre, H. Seaweed Proteins-How to Get to Them? Effects of Processing on Nutritional Value, Bioaccessibility and Extractability. Ph.D. Thesis, Faculty of Biosciences, Fisheries and Economics, Norwegian College of Fishery Science, Tromsø, Norway, 2015.
67. Domozych, D. Algal Cell Walls. *eLS* **2019**, 1–11. [\[CrossRef\]](#)
68. Weinbreck, F.; Nieuwenhuijse, H.; Robijn, G.; de Kruif, C. Complexation of Whey Proteins with Carrageenan. *J. Agric. Food Chem.* **2004**, *52*, 3550–3555. [\[CrossRef\]](#) [\[PubMed\]](#)
69. Veide Vilg, J.; Undeland, I. PH-Driven Solubilization and Isoelectric Precipitation of Proteins from the Brown Seaweed *Saccharina latissima*—Effects of Osmotic Shock, Water Volume and Temperature. *J. Appl. Phycol.* **2017**, *29*, 585–593. [\[CrossRef\]](#) [\[PubMed\]](#)
70. Kadam, S.; Tiwari, B.; O'Donnell, C. Application of Novel Extraction Technologies for Bioactives from Marine Algae. *J. Agric. Food Chem.* **2013**, *61*, 4667–4675. [\[CrossRef\]](#)
71. Abreu, T.; Monteiro, V.; Martins, A.; Teles, F.; Rivanor, R.; Mota, É.; Macedo, D.S.; de Vasconcelos, S.; Honório, J.E.R., Jr.; Benevides, N. Involvement of the Dopaminergic System in the Antidepressant-like Effect of the Lectin Isolated from the Red Marine Alga *Solieria filiformis* in Mice. *Int. J. Biol. Macromol.* **2018**, *111*, 534–541. [\[CrossRef\]](#) [\[PubMed\]](#)
72. Vasconcelos, M.; Arruda, F.; Carneiro, V.; Silva, H.; Nascimento, K.; Sampaio, A.; Cavada, B.; Teixeira, E.; Henriques, M.; Pereira, M. Effect of Algae and Plant Lectins on Planktonic Growth and Biofilm Formation in Clinically Relevant Bacteria and Yeasts. *BioMed Res. Int.* **2014**, *2014*, 365272. [\[CrossRef\]](#)
73. Leite, Y.; Silva, L.; Amorim, R.; Freire, E.; Jorge, D.; Grangeiro, T.; Benevides, N. Purification of a Lectin from the Marine Red Alga *Gracilaria ornata* and Its Effect on the Development of the Cowpea Weevil *Callosobruchus maculatus* (Coleoptera: Bruchidae). *Biochim. Biophys. Acta Gen. Subj.* **2005**, *1724*, 137–145. [\[CrossRef\]](#)
74. Fleurence, J.; Massiani, L.; Guyader, O.; Mabeau, S. Use of Enzymatic Cell Wall Degradation for Improvement of Protein Extraction from *Chondrus crispus*, *Gracilaria verrucosa* and *Palmaria palmata*. *J. Appl. Phycol.* **1995**, *7*, 393–397. [\[CrossRef\]](#)
75. Vásquez, V.; Martínez, R.; Bernal, C. Enzyme-Assisted Extraction of Proteins from the Seaweeds *Macrocystis pyrifera* and *Chondracanthus chamosoi*: Characterization of the Extracts and Their Bioactive Potential. *J. Appl. Phycol.* **2019**, *31*, 1999–2010. [\[CrossRef\]](#)
76. Burlot, A.; Bedoux, G. Response Surface Methodology for Enzyme-Assisted Extraction of Water-Soluble Antiviral Compounds from the Proliferative Macroalga *Solieria chordalis*. *Enzym. Eng.* **2016**, *5*, 1000148. [\[CrossRef\]](#)
77. Harnedy, P.; Fitzgerald, R. Extraction of Protein from the Macroalga *Palmaria palmata*. *LWT Food Sci. Technol.* **2013**, *51*, 375–382. [\[CrossRef\]](#)
78. Vilku, K.; Mawson, R.; Simons, L.; Bates, D. Applications and Opportunities for Ultrasound Assisted Extraction in the Food Industry—A Review. *Innov. Food Sci. Emerg. Technol.* **2008**, *9*, 161–169. [\[CrossRef\]](#)
79. Vilku, K.; Manasseh, R.; Mawson, R.; Ashokkumar, M. Ultrasonic Recovery and Modification of Food Ingredients. In *Ultrasound Technologies for Food and Bioprocessing*; Food Engineering Series; Springer: New York, NY, USA, 2011; pp. 345–368. [\[CrossRef\]](#)
80. Grosso, C.; Valentão, P.; Ferreres, F.; Andrade, P. Alternative and Efficient Extraction Methods for Marine-Derived Compounds. *Mar. Drugs* **2015**, *13*, 3182–3230. [\[CrossRef\]](#)
81. Wu, H.; Hulbert, G.; Mount, J. Effects of Ultrasound on Milk Homogenization and Fermentation with Yogurt Starter. *Innov. Food Sci. Emerg. Technol.* **2000**, *1*, 211–218. [\[CrossRef\]](#)
82. Chemat, F.; Zill-e-Huma; Khan, M.K. Applications of Ultrasound in Food Technology: Processing, Preservation and Extraction. *Ultrason. Sonochem.* **2011**, *18*, 813–835. [\[CrossRef\]](#)
83. Mittal, R.; Tavanandi, H.; Mantri, V.; Raghavarao, K. Ultrasound Assisted Methods for Enhanced Extraction of Phycobiliproteins from Marine Macro-Algae, *Gelidium pusillum* (Rhodophyta). *Ultrason. Sonochem.* **2017**, *38*, 92–103. [\[CrossRef\]](#)

84. Fleurence, J.; le Coeur, C.; Mabeau, S.; Maurice, M.; Landrein, A. Comparison of Different Extractive Procedures for Proteins from the Edible Seaweeds *Ulva rigida* and *Ulva rotundata*. *J. Appl. Phycol.* **1995**, *7*, 577–582. [\[CrossRef\]](#)
85. Harrysson, H.; Hayes, M.; Eimer, F.; Carlsson, N.; Toth, G.; Undeland, I. Production of Protein Extracts from Swedish Red, Green, and Brown Seaweeds, *Porphyra umbilicalis* Kützinger, *Ulva lactuca* Linnaeus, and *Saccharina latissima* (Linnaeus) J. V. Lamouroux Using Three Different Methods. *J. Appl. Phycol.* **2018**, *30*, 3565–3580. [\[CrossRef\]](#)
86. Robin, A.; Kazir, M.; Sack, M.; Israel, A.; Frey, W.; Mueller, G.; Livney, Y.; Golberg, A. Functional Protein Concentrates Extracted from the Green Marine Macroalga *Ulva* sp. by High Voltage Pulsed Electric Fields and Mechanical Press. *ACS Sustain. Chem. Eng.* **2018**, *6*, 13696–13705. [\[CrossRef\]](#)
87. Stichler, O. Natural Product Isolation. *Nat. Prod. Rep.* **2008**, *25*, 517–554. [\[CrossRef\]](#)
88. Rostagno, M.; Villares, A.; Guillaumon, E.; García-Lafuente, A.; Martínez, J. Sample Preparation for the Analysis of Isoflavones from Soybeans and Soy Foods. *J. Chromatogr. A* **2009**, *1216*, 2–29. [\[CrossRef\]](#) [\[PubMed\]](#)
89. Romanik, G.; Gilgenast, E.; Przyjazny, A.; Kamiński, M. Techniques of Preparing Plant Material for Chromatographic Separation and Analysis. *J. Biochem. Biophys. Methods* **2007**, *70*, 253–261. [\[CrossRef\]](#) [\[PubMed\]](#)
90. Pathak, Y.; Wang, L. Advances in Extraction of Plant Products in Nutraceutical Processing. In *Handbook of Nutraceuticals Volume II*; CRC Press: Boca Raton, FL, USA, 2011; pp. 15–52. [\[CrossRef\]](#)
91. Lu, J.; Feng, X.; Han, Y.; Xue, C. Optimization of Subcritical Fluid Extraction of Carotenoids and Chlorophyll a from *Laminaria japonica* Aresch by Response Surface Methodology. *J. Sci. Food Agric.* **2014**, *94*, 139–145. [\[CrossRef\]](#)
92. Herrero, M.; Castro-Puyana, M.; Mendiola, J.; Ibañez, E. Compressed Fluids for the Extraction of Bioactive Compounds. *TrAC Trends Anal. Chem.* **2013**, *43*, 67–83. [\[CrossRef\]](#)
93. Quitain, A.; Kai, T.; Sasaki, M.; Goto, M. Supercritical Carbon Dioxide Extraction of Fucoxanthin from *Undaria pinnatifida*. *J. Agric. Food Chem.* **2013**, *61*, 5792–5797. [\[CrossRef\]](#)
94. Careri, M.; Furlattini, L.; Mangia, A.; Musci, M.; Anklam, E.; Theobald, A.; von Holst, C. Supercritical Fluid Extraction for Liquid Chromatographic Determination of Carotenoids in *Spirulina pacifica* Algae: A Chemometric Approach. *J. Chromatogr. A* **2001**, *912*, 61–71. [\[CrossRef\]](#)
95. Mendes, R.; Nobre, B.; Cardoso, M.; Pereira, A.; Palavra, A. Supercritical Carbon Dioxide Extraction of Compounds with Pharmaceutical Importance from Microalgae. *Inorg. Chim. Acta* **2003**, *356*, 328–334. [\[CrossRef\]](#)
96. Khaw, K.; Parat, M.; Shaw, P.; Falconer, J. Solvent Supercritical Fluid Technologies to Extract Bioactive Compounds from Natural Sources: A Review. *Molecules* **2017**, *22*, 1186. [\[CrossRef\]](#) [\[PubMed\]](#)
97. Manjare, S.; Dhingra, K. Supercritical Fluids in Separation and Purification: A Review. *Mater. Sci. Energy Technol.* **2019**, *2*, 463–484. [\[CrossRef\]](#)
98. Gallego, R.; Bueno, M.; Herrero, M. Sub- and Supercritical Fluid Extraction of Bioactive Compounds from Plants, Food-by-Products, Seaweeds and Microalgae—An Update. *Trends Anal. Chem.* **2019**, *116*, 198–213. [\[CrossRef\]](#)
99. Mohd, N.; Draman, S.; Salleh, M.; Yusof, N. Dissolution of Cellulose in Ionic Liquid: A Review. In *AIP Conference Proceedings*; American Institute of Physics Inc.: College Park, MD, USA, 2017; Volume 1809. [\[CrossRef\]](#)
100. Swatloski, R.; Spear, S.; Holbrey, J.; Rogers, R. Dissolution of Cellulose with Ionic Liquids. *J. Am. Chem. Soc.* **2002**, *124*, 4974–4975. [\[CrossRef\]](#) [\[PubMed\]](#)
101. Tang, B.; Bi, W.; Tian, M.; Row, K. Application of Ionic Liquid for Extraction and Separation of Bioactive Compounds from Plants. *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* **2012**, *904*, 1–21. [\[CrossRef\]](#)
102. Angell, A.; Paul, N.; de Nys, R. A Comparison of Protocols for Isolating and Concentrating Protein from the Green Seaweed *Ulva ohnoi*. *J. Appl. Phycol.* **2017**, *29*, 1011–1026. [\[CrossRef\]](#)
103. Kumar, P.; Sharma, N.; Ranjan, R.; Kumar, S.; Bhat, Z.; Jeong, D. Perspective of Membrane Technology in Dairy Industry: A Review. *Asian-Australas. J. Anim. Sci.* **2013**, *26*, 1347–1358. [\[CrossRef\]](#) [\[PubMed\]](#)
104. Shao, P.; Chen, M.; Pei, Y.; Sun, P. In Intro Antioxidant Activities of Different Sulfated Polysaccharides from Chlorophytan Seaweeds *Ulva fasciata*. *Int. J. Biol. Macromol.* **2013**, *59*, 295–300. [\[CrossRef\]](#)
105. Araki, C. Some Recent Studies on the Polysaccharides of Agarophytes. In *Proceedings of the Fifth International Seaweed Symposium*, Halifax, NS, Canada, 25–28 August 1966; Elsevier: Amsterdam, The Netherlands, 1966; pp. 3–17. [\[CrossRef\]](#)
106. Saluri, M.; Kaldmäe, M.; Tuvikene, R. Extraction and Quantification of Phycobiliproteins from the Red Alga *Furcellaria lumbricalis*. *Algal Res.* **2019**, *37*, 115–123. [\[CrossRef\]](#)
107. Senthilkumar, N.; Suresh, V.; Thangam, R.; Kurinjimalar, C.; Kavitha, G.; Murugan, P.; Rengasamy, R. Isolation and Characterization of Macromolecular Protein R-Phycocerythrin from *Portieria hornemannii*. *Int. J. Biol. Macromol.* **2013**, *55*, 150–160. [\[CrossRef\]](#)
108. Niu, J.; Xu, M.; Wang, G.; Zhang, K.; Peng, G. Comprehensive Extraction of Agar and R-Phycocerythrin from *Gracilaria lemaneiformis* (Bangiales, Rhodophyta). *Indian J. Geo-Mar. Sci.* **2013**, *42*, 21–28.
109. Jong, L.; Thien, V.; Yong, Y.; Rodrigues, K.; Yong, W. Micropropagation and Protein Profile Analysis by SDS-PAGE of *Gracilaria changii* (Rhodophyta, Solieriaceae). *Aquac. Rep.* **2015**, *1*, 10–14. [\[CrossRef\]](#)
110. Rosni, M.; Fisal, A.; Azwan, A.; Chye, F.Y.; Matanjun, P. Crude Proteins, Total Soluble Proteins, Total Phenolic Contents and SDS-PAGE Profile of Fifteen Varieties of Seaweed from Semporna, Sabah, Malaysia. *Int. Food Res. J.* **2015**, *22*, 1483–1493.

111. Tsuji, A.; Kuwamura, S.; Shirai, A.; Yuasa, K. Identification and Characterization of a 25 KDa Protein That Is Indispensable for the Efficient Saccharification of *Eisenia bicyclis* in the Digestive Fluid of *Aplysia kurodai*. *PLoS ONE* **2017**, *12*, e0170669. [[CrossRef](#)] [[PubMed](#)]
112. Garcia-Vaquero, M.; Lopez-Alonso, M.; Hayes, M. Assessment of the Functional Properties of Protein Extracted from the Brown Seaweed *Himanthalia elongata* (Linnaeus) S. F. Gray. *Food Res. Int.* **2017**, *99*, 971–978. [[CrossRef](#)] [[PubMed](#)]
113. Go, H.; Hwang, H.; Nam, T. Glycoprotein Extraction from *Laminaria japonica* Promotes IEC-6 Cell Proliferation. *Int. J. Mol. Med.* **2009**, *24*, 819–824. [[CrossRef](#)]
114. Choi, Y.; Yamaguchi, K.; Oda, T.; Nam, T. Chemical and Mass Spectrometry Characterization of the Red Alga *Pyropia yezoensis* Chemoprotective Protein (PYP): Protective Activity of the N-Terminal Fragment of PYP1 against Acetaminophen-Induced Cell Death in Chang Liver Cells. *Int. J. Mol. Med.* **2015**, *35*, 271–276. [[CrossRef](#)]
115. Fernández-Segovia, I.; Lerma-García, M.; Fuentes, A.; Barat, J. Characterization of Spanish Powdered Seaweeds: Composition, Antioxidant Capacity and Technological Properties. *Food Res. Int.* **2018**, *111*, 212–219. [[CrossRef](#)]
116. Vieira, E.; Soares, C.; Machado, S.; Correia, M.; Ramalhosa, M.; Oliva-Teles, M.; Paula Carvalho, A.; Domingues, V.; Antunes, F.; Oliveira, T.; et al. Seaweeds from the Portuguese Coast as a Source of Proteinaceous Material: Total and Free Amino Acid Composition Profile. *Food Chem.* **2018**, *269*, 264–275. [[CrossRef](#)]
117. Kazir, M.; Abuhassira, Y.; Robin, A.; Nahor, O.; Luo, J.; Israel, A.; Golberg, A.; Livney, Y. Extraction of Proteins from Two Marine Macroalgae, *Ulva* sp. and *Gracilaria* sp., for Food Application, and Evaluating Digestibility, Amino Acid Composition and Antioxidant Properties of the Protein Concentrates. *Food Hydrocoll.* **2019**, *87*, 194–203. [[CrossRef](#)]
118. Abdollahi, M.; Axelsson, J.; Carlsson, N.; Nylund, G.; Albers, E.; Undeland, I. Effect of Stabilization Method and Freeze/Thaw-Aided Precipitation on Structural and Functional Properties of Proteins Recovered from Brown Seaweed (*Saccharina latissima*). *Food Hydrocoll.* **2019**, *96*, 140–150. [[CrossRef](#)]
119. Kumar, K.; Ganesan, K.; Selvaraj, K.; Rao, P. Studies on the Functional Properties of Protein Concentrate of *Kappaphycus alvarezii* (Doty) Doty—An Edible Seaweed. *Food Chem.* **2014**, *153*, 353–360. [[CrossRef](#)] [[PubMed](#)]
120. Romarís-Hortas, V.; Bermejo-Barrera, P.; Moreda-Piñeiro, A. Ultrasound-Assisted Enzymatic Hydrolysis for Iodinated Amino Acid Extraction from Edible Seaweed before Reversed-Phase High Performance Liquid Chromatography-Inductively Coupled Plasma-Mass Spectrometry. *J. Chromatogr. A* **2013**, *1309*, 33–40. [[CrossRef](#)] [[PubMed](#)]