Techno-Functional Properties of Crude Extracts from the Green Microalga *Tetraselmis suecica*

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**ABSTRACT:** A mild fractionation process to extract functional biomolecules from green microalgae was implemented. The process includes bead milling, centrifugation, and filtration with several membrane cut-offs. For each fraction, the corresponding composition was measured, and the surface activity and gelation behavior were determined. A maximum protein yield of 12% was obtained in the supernatant after bead milling and between 3.2 and 11.7% after filtration. Compared to whey protein isolate, most of the algae fractions exhibited comparable or enhanced functionality. Surface activity for air–water and oil–water interfaces and gelation activities were notably superior for the retentate fractions compared to the permeates. It is proposed that such functionality in the retentates is due to the presence of hydrophobic compounds and molecular complexes exhibiting a similar behavior as Pickering particles. We demonstrated that excellent functionality can be obtained with crude fractions, requiring minimum processing and, thus, constituting an interesting option for commercial applications.

**KEYWORDS:** crude extract, bead milling, filtration, surface activity, gelation

1. INTRODUCTION

Algae have been recognized as a promising renewable feedstock for the production of fuels and bulk chemicals, pigments, and particularly food and feed ingredients.\(^1\) To obtain such products, an intricate series of unit operations are often needed, involving cell disintegration, extraction, and purification. An algae biorefinery is therefore associated with multiple downstream processing steps that result in several highly pure products.\(^2\) For some applications, however, product functionality must be the determining criterion, rather than product purity.\(^3\) The functional properties of a certain product (e.g., foaming, emulsification, and gelation) are determined by the presence of proteins, carbohydrates, and lipids and the interactions among them.\(^3\) It is therefore expected that complex mixtures also show certain functionality. In other words, impure fractions can be potentially marketed as functional ingredients.

The functionality of algae proteins has been investigated in several publications. Excellent gelling properties were observed for proteins extracted from the cyanobacteria *Arthrospira platensis*.\(^4\) A soluble protein isolate (ASPI) from the microalga *Tetraselmis sp.* was prepared by Schwenzfeier et al.\(^5\) The ASPI, containing 64% proteins and 24% carbohydrates, showed complete solubility at a pH above 5.5, the formation of stable emulsions at pH 5–7,\(^6\) and superior foam stability at pH 5–7, compared to whey and egg protein isolates.\(^7\) The authors argued that the presence of charged sugars contributed to the foaming and emulsifying properties of the algae protein isolate.\(^7\) Proteins from the microalga *Chlorella vulgaris* were extracted after a process of homogenization, pH shift, and ultrafiltration.\(^9\) Only the permeate fractions obtained under neutral conditions showed higher emulsifying capacity and stability than commercial sodium caseinate and soy protein isolate. Similarly, water-soluble proteins from *Haematococcus pluvialis* were extracted using high-pressure homogenization, centrifugation, and pH shift. The resulting supernatants were rich in proteins (26–44 dw %), carbohydrates, and lipids and exhibited superior emulsifying stability and activity index in comparison to sodium caseinate. Emulsification capacity, however, was lower.\(^10\)

A protein isolate (70 dw % proteins) obtained from *Arthrospira platensis* was evaluated for several functional properties.\(^11\) It was found that emulsification and foaming are highly dependent on the pH and correlate directly with protein solubility. Under the presence of a plasticizer, the fractions were able to form stable gels. Isoelectric precipitation was applied to extract proteins from bead-milled *Nannochloropsis oculata*.\(^12\) The extract, containing 23% proteins and 15% lipids (dw), was proposed as an interesting functional ingredient for food and feed. Extraction and precipitation of proteins from *Nannochloropsis spp.* after thermal treatment and pH shift were reported by Gerde et al.\(^13\) Although the extraction conditions were harsh, the authors pointed out that the high degree of glycosylation of the protein extract could have led to unique functional properties. Waghmare et al.\(^14\)
employed a three-phase system to concentrate proteins from *Chlorella pyrenoidosa* and obtained a concentrate with 78% (dw) proteins, exhibiting excellent foaming and good oil holding capacities.

The present study presents an overview of the functional activity (surface activity and gelation behavior) of extracts obtained under mild conditions from the marine microalga *Tetraselmis suecica*. The fractionation process consists of bead milling, centrifugation, and filtration, which are simple and standard technologies in downstream processing and thus with high potential to be scalable. The effect of the membrane cutoff on the composition and functionality of the permeates and retentates was also investigated. The main objective of this research was to demonstrate a simple fractionation strategy to recover algae fractions and to show that crude extracts display comparable or superior functionality to commercial protein isolates.

### 2. MATERIALS AND METHODS

#### 2.1. Algae Cultivation and Harvesting. *Tetraselmis suecica* (UTEX LB2286, University of Texas Culture Collection of Algae, USA) was cultivated and harvested as described by Postma et al. To summarize, dry weight was estimated by burning a known amount of freeze-dried biomass in an oven at 575 °C and regarding the remaining material as ash. Total lipids were measured with the method of Folch, and starch with the total starch assay kit of Megazyme. Total ash was determined gravimetrically by burning a known amount of freeze-dried biomass in an oven at 575 °C and regarding the remaining material as ash.

#### 2.2. Preparation of Algae Fractions. A simple fractionation process is proposed, involving the steps of bead milling, centrifugation, and filtration (Figure 1). After separation, every fraction (crude extract, solids, permeate, and retentate) was collected and analyzed independently.

![Figure 1. Fractionation strategy of bead-milled algae suspensions.](Image)

**Bead Milling and Centrifugation.** Disruption experiments were conducted in a horizontal 0.075 L bead mill (Dyno-Mill Research, Willy A. Bachofen AF Maschinenfabrik, Switzerland) operated in batch recirculation mode. Algae suspensions containing ~100 g L⁻¹ of biomass were prepared in phosphate saline buffer pH 7 (1.54 mM KH₂PO₄, 2.71 mM Na₂HPO₄·2H₂O, 155.2 mM NaCl). All runs were conducted for 1 h, under the same conditions as presented before, except bead size, which was kept constant at 0.4 mm. Bead-milled suspensions were centrifuged at 20 000g and 20 °C for 30 min, and the supernatants and pellets were stored separately at −20 °C until further use.

**Filtration.** Ultrafiltration experiments were conducted on a LabscaleTM TFF system (Millipore, Bellerica, MA) filled with membrane cassettes with a filtration area of 50 cm² and cut-offs of 300 kDa or 10 kDa (Pellicon XL Ultrafiltration Ultraceil) at a fixed average transmembrane pressure (TMP) of 2.07 bar. Microfiltration was performed by manually pressing feed (crude extract) through 0.45 μm dead-end cellulose filters (Sartorius). Permeates and retentates were stored at −20 °C until analysis.

#### 2.3. Analytical Methods. Biomass Characterization.** Dry weight (DW), proteins, carbohydrates, and starch analyses were conducted as described by Postma et al. To summarize, dry weight was estimated gravimetrically, and proteins and carbohydrates were measured with the methods of Lowry and Dubois, respectively. Total lipids were measured with the method of Folch and starch with the total starch assay kit of Megazyme. Total ash was determined gravimetrically by burning a known amount of freeze-dried biomass in an oven at 575 °C and regarding the remaining material as ash.

**Mass Yields.** Mass yields per component (Y₁) were estimated according to

\[
y_1 = \frac{m_i}{m_b} \times 100
\]

where \( m_i \) is the mass of component \( i \) (protein, carbohydrates, lipids, ash, and starch). Subscripts \( j \) and \( b \) refer to each fraction evaluated (crude extract, permeate, filtrate, etc.) and initial biomass, respectively.

**Acrylamide Native Gel Electrophoresis.** Protein samples were prepared in Milli-Q water and diluted with native buffer (Biorad) at a ratio 1:0.8 v/v. An amount of 25 μL of the resulting solution was loaded per lane in a 4–20% Criterion TGX gel (Biorad). NativeMark (Life Technologies) was used as marker. Electrophoresis was run at 125 V constant for 75 min using tris-glycine (Biorad) as running buffer. Staining of the gels was performed with Bio-Safe Coomassie blue (Biorad) for 2 h. Gels were left overnight after abundant washing with demineralized water to further develop the bands before scanning.

**Particle Size.** Particle size distributions of solutions containing algae extracts (0.1% w/v protein basis) were determined using a Nanosizer-Zetasizer Malvern ZEN 5600SN (Malvern Instruments Ltd., Malvern, UK) at room temperature and pH 7.

**2.4. Technofunctional Properties.** Protein Solutions. Before determining technofunctional properties, samples were lyophilized for at least 27 h using a Sublimator 2 × 3 × 3 (Zirbus Technology GmbH) and stored airtight at room temperature until use. The corresponding amount of algae fraction was weighted and dissolved in distilled water in order to obtain a desired protein concentration. The resulting solution was adjusted to pH 7 with NaOH 0.1 M before each analysis.

**Whey Protein Isolate (WPI).** Whey protein isolate (BiPro, Divosco Foods international) containing 97.6% protein and 2% ash (DW) and 5% moisture content was used as the reference commercial standard.

**Surface Activity.** The ability of the fractions to influence surface tension was determined by recording the interfacial tension of static-controlled droplets using an automated drop tensiometer (ADT Tracker, Tectis Scientific, France). The ADT measures surface tension according to the Young–Laplace theory. Foaming and emulsification behavior were derived from static drop experiments as presented below. All experiments were conducted at room temperature.

**Foaming.** Surface activity for the air–water interface was studied from air–water droplets. Each droplet was formed with 11 μL of suspension containing 0.1% protein (w/v). The droplet was kept hanging from a needle while subjected to a stream of saturated air flowing vertically in a 5 mL cuvette. Surface tension of the droplet was recorded for a period of 36 min.

**Emulsification.** Studies of surface activity for oil–water interface were conducted on a 20 μL static drop of hexadecane (Anhydrous, >99%, Sigma-Aldrich) submerged in 5 mL of 0.1% protein solution (w/v). Surface tension was recorded for 60 min. After equilibrium is reached, perturbations on the droplet’s volume were enforced by adding 1 μL of solvent, 5 times in 10 s, ensuring variations in surface area lower than 10%. Fourier analysis was conducted on the dilation data, resulting in the elastic modulus \( \varepsilon \) (mN m⁻²).

**Gelation.** Gelation tests were conducted according to Martin et al., using an Anton Paar MCR 302 (Modular Compact Rheometer) with a heating rate of 5 °C min⁻¹ in the range 25 to 95 °C.

**2.5. Statistics.** All experiments were conducted in duplicates from independent experiments. Statistical analysis at 95% confidence level was conducted using R (V 3.2.2). Significance was evaluated applying one-way ANOVA. To compare significantly different means, a t test or a Tukey’s Honest Significant Test (HSD) was applied.
3. RESULTS AND DISCUSSION

3.1. Fractionation Process. The complete mass balance and corresponding compositions and mass yields per component, according to the fractionation process depicted in Figure 1, are presented in Table 1 and Figure 2A. The carbohydrate content in the initial biomass (41% dw) was significantly higher compared to the values reported by Schwenzfeier et al. for Tetraselmis sp. (24% dw), while the protein content was similar (~37% dw). The high content of carbohydrates can be due to the accumulation of starch granules and seasonal variation as noted by Michels et al. for cultures of Tetraselmis suecica maintained in greenhouses.

During bead milling, the shear caused by bead-bead collisions leads to the release of intracellular components. Proteins are released quickly, reaching a maximum concentration at short milling times. Carbohydrates, on the contrary, display a gradual increasing trend as noted in our previous study. After bead milling, over 30% of the total sugars and only 12% of the proteins were found in the soluble phase or “Crude Extract (CE)” (Figure 2B). This indicates that only a small fraction of the total proteins in T. suecica is soluble and can be extracted in the aqueous phase after complete mechanical disintegration. Postma et al. reported yields of soluble proteins of approximately 20% for Tetraselmis species after bead milling. This higher yield can be due to differences in biomass composition and in the calculation method, as we are reporting mass yields according to eq 1. The insoluble phase (solids) contains almost equal proportions of proteins, carbohydrates, and lipids, in addition to 5.3 ± 0.9% (dw) ash (Figure 2A). It constitutes an interesting material for the preparation of feed formulations for livestock, poultry, and aquaculture.

The effect of the membrane cutoff on the fractionation yields and functionality was further investigated. Sequential filtration has been applied for algae biorefinery, but the study of the functionality of the resulting fractions remains elusive. For each membrane cutoff, the content of proteins, carbohydrates, lipids, and ash was quantified, and the results are presented in Table 1. The corresponding mass yields are also given. The permeate fraction after microfiltration (0.45 μm membrane) resulted in the highest yields for all components. This is anticipated as this membrane removes only large particles, yielding a permeate containing nearly 97% of the total feed. Unexpectedly, the protein yield in the permeate of the 300 kDa membrane doubles that of the 300 kDa. This can be due to fouling for the 300 kDa membrane, preventing a significant fraction of proteins to migrate to the permeate. The same phenomenon was observed by Safi et al., who noted a higher degree of membrane fouling for higher cutoffs and attributed this to the formation of polarization layers and to the adsorptive fouling during the filtration of algae suspensions. Such fouling can also explain why the retentate of the 300 kDa membrane shows a higher amount of total lipids compared with the 10 kDa.

The permeates of the 300 and 10 kDa appeared clear, indicating complete removal of pigments. This was also observed in other anstudy for extracts from Nannochloropsis gaditana using polyethersulfone membranes of 1000, 500, and 300 kDa. Pigments are recovered in the retentate phase due mainly to the hydrophilic nature of the membrane materials used in both studies. Regarding starch, we measured concentrations in the range 1.1–1.9 g kg⁻¹ in both the

### Table 1. Compositions [g kg⁻¹] of Fractions after Bead Milling and Filtration

<table>
<thead>
<tr>
<th>Component</th>
<th>Feed</th>
<th>Pellet</th>
<th>Filtration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>bead milling</td>
<td>300 kDa</td>
<td>10 kDa</td>
</tr>
<tr>
<td>Dry mass</td>
<td>129.2 ± 6.6</td>
<td>16.5 ± 0.5</td>
<td>18.5 ± 0.4</td>
</tr>
<tr>
<td>Protein</td>
<td>40.4 ± 2.0</td>
<td>8.9 ± 0.3</td>
<td>7.2 ± 0.2</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>47.2 ± 2.0</td>
<td>11.0 ± 0.7</td>
<td>9.8 ± 0.3</td>
</tr>
<tr>
<td>Lipids</td>
<td>20.4 ± 1.0</td>
<td>4.8 ± 0.3</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>Ash</td>
<td>20.3 ± 1.0</td>
<td>4.8 ± 0.3</td>
<td>3.8 ± 0.2</td>
</tr>
<tr>
<td>Starch</td>
<td>20.3 ± 1.0</td>
<td>4.8 ± 0.3</td>
<td>3.8 ± 0.2</td>
</tr>
</tbody>
</table>

The data presented are the average of duplicates and corresponding standard deviations. Values in parentheses indicate mass yield per compound—per permeate and retentates, respectively (g ± 0.05).

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permeate and retentate fractions (Table 1). This contradicts the results of Safi et al.,23,24 who reported complete retention of starch for membranes ranging from 1000 to 10 kDa. This difference can be due to a lesser extent of fouling for the cellulose-based membranes used in our research compared to polyethersulfone-based membranes used by Safi and co-workers, allowing starch fragments to also migrate to the permeate phase.

Besides a strong green color, the retentate fractions showed a significantly higher content of proteins, carbohydrates, and lipids (Table 1). This suggests that proteins are associated with pigments and polysaccharides. In fact, it has been reported that proteins in green microalgae are often covalently bound to lipids,25 polysaccharides, sugars,5,8,26 and pigments,27 forming molecular complexes that can easily be retained by membranes during ultrafiltration.

The fractionation process presented in this investigation was conducted under mild conditions (room temperature and native pH of 5.7 ± 0.2) and without the addition of chemicals. Native gel electrophoresis (Figure 3) shows the expected bands for T. suecica15 and demonstrates that after bead milling and filtration the main protein bands are maintained. In addition, it is confirmed that the permeate of the 10 kDa only contains low molecular weight proteins. A maximum overall total protein yield of 6.1% was observed after filtration (Table 1). This is comparable with a 7% yield reported for Tetraselmis sp. under a process involving bead milling, dialysis, chromatography, and precipitation.5 On the contrary, Ursu et al.7 found a yield of 87% for Chlorella vulgaris after filtration over a 300 kDa membrane. The authors attributed this to the fact that proteins from the algae extracts exist as large macromolecular aggregates with molecular weights above 670 kDa, and thus the majority of the proteins is retained. The corresponding protein yields for the filtration step in the present investigation are 40.1% and 26.9% for the retentates of the 300 and 10 kDa membranes, respectively. The lower yields are an indication of a more diverse range of proteins and macromolecular complexes in the extracts from T. suecica. Such diversity may lead to a richer technical functionality.

3.2. Technofunctional Properties. 3.2.1. Surface Activity: Foaming and Emulsification. Surface activity—foaming and emulsification—refers to the ability of certain compounds to form and stabilize air−water (awi) or oil−water (owi) interfaces. Such stabilization takes place due to the formation of network-like structures around a clean surface, which effectively lowers its surface tension. This requires surface active molecules to be soluble, to diffuse to, and to adsorb on an interface.28 Furthermore, molecular rearrangements and interactions among molecules adsorbed on the surface also lead to variations of the surface tension.29 In this regard, functionality is not limited to proteins but can be enhanced by the presence and chemical nature of other biomolecules and their ability to interact.3

The dynamic surface tension (γ [mN m−1]) of samples containing extracts from T. suecica and whey protein isolate (WPI) as reference protein is presented in Figure 3A for awi and Figure 3B for owi. For both cases, the surface tension decreases sharply and reaches slowly an equilibrium level. This...
behavior is typical for surface active molecules and reflects the basic mechanisms mentioned before. For a theoretical treatment of the experimental data presented in Figure 3, the reader is referred to the Supporting Information.

The surface activity of alga extracts in awi showed a comparable or superior performance to samples prepared with WPI. This can be seen in Figure 3A by comparing the slopes (reflecting the rates of diffusion, adsorption and stabilization) and the surface tension at equilibrium conditions, which reflects the extent of surface activity. The retentate fractions from the 10 and 300 kDa membranes resulted in the strongest activities. On the contrary, the permeate from the 10 kDa membrane showed the poorest performance. This clearly demonstrates the effect of the fractionation strategy. The retentate fractions are rich in pigments and lipids and contain larger particles and a wider range of proteins (Figure 4). On the contrary, the permeate fractions are depleted of pigments, contain only small particles, and, for the case of the 10 kDa membrane, lack large molecular weight proteins (Figure 4A). The presence of larger macromolecular complexes appears to favor the stabilization process. It is therefore not surprising that the alga extracts, containing a mixture of compounds, presented a higher activity than a pure protein isolate. This has been attributed not only to proteins and glycoproteins but also to charged sugars and pigments.

The surface activity in owi presented a similar behavior: a sharp decline of γ followed by a slow decrease to reach a plateau phase (Figure 3B). All samples containing alga extracts showed a comparable performance as WPI. Other studies have also found a similar or superior emulsification activity of algae proteins in comparison with commercial protein isolates. The superior performance has been attributed to the activity of the proteins present in the extracts, but also to the interactions and favorable effect of other biomolecules present in the extract such as sugars, chlorophyll and lipids. Emulsion stabilization takes place because of the development of steric forces around the surfaces which limit the extent of coalescence and emulsion degradation.

For the retentate fractions (10 and 300 kDa) measurements of γ could not continue beyond 1500 s (data not shown) due to the sudden detachment of the hexadecane drop from the measurement device. This suggests a remarkable surface stabilization, probably brought about by the presence of pigments and higher lipid content (Table 1) which allows a

Figure 4. (A) Native gel analysis of fractions before and after filtration (M: Marker; CE: Crude Extract; R: Retentate; P: Permeate; Mi: 0.45 μm microfiltration; F3: 300 kDa filtration; F1: 10 kDa filtration). Dotted arrows and squares indicate the expected band of Rubisco (∼540 kDa). (B) Average particle size for several alga fractions after ultrafiltration.

Figure 5. (A) Storage modulus (\(G'\) [Pa]) as a function of time and temperature (—) for algae fractions: black — CE 10%, —X— 0.45 μm P 10%, gray —· CE 5%, black —· 300 kDa R 5%, gray —· 300 kDa P 5%, black — — 10 kDa R 5%, gray — — 10 kDa P 5%. (B) Comparison of algae fractions: □□□□ CE 10%, ○○○○ 0.45 μm P 10%, and commercial proteins: •••• 2.5% RuBisCO, — — 10% EWP, · · · · 12.5% WPI.
stronger interaction with the hexadecane. For the remaining fractions, surface activity was further studied by imposing periodic expansions and compressions on the droplet’s surface area (Figure 3B). As can be seen, all samples recovered their original surface tension without appreciable deformation, indicating a high degree of stability. A measure of such stability during perturbation is obtained with the elastic modulus \( \varepsilon \). The values of \( \varepsilon \) for samples containing alga extracts varied between 35 and 41 mN \( \text{m}^{-1} \), while that for WPI was nearly 40 mN \( \text{m}^{-1} \). This elastic behavior is typical of molecules which can store energy, such as proteins.\(^{30}\)

Contrary to our findings, Ursu et al.\(^9\) observed better emulsification activity for the permeates of a 300 kDa (polyethersulfone) filtration process. The authors argued that after the extraction process proteins were denatured and formed aggregates, which were later recovered in the retentate fractions. Such aggregates therefore displayed inferior functionality. As indicated by native gel analysis (Figure 4A), the fractionation process employed in the present research did not lead to appreciable protein denaturation nor aggregation, and thus, both fractions are enriched with functional molecules.

3.2.2. Gelation. The textural attributes of several food products result from the development of stable gels. In general terms, gels are formed after a two-step process. In the first step the functional groups of the active molecules are exposed due to thermal or chemical denaturation. Later, the exposed functional groups interact with specific regions of neighboring molecules, creating a network like structure. Furthermore, cross-links are developed, leading to a three-dimensional assembly with specific viscoelastic properties.\(^31\) Only few studies have addressed the gelation properties of algal proteins. In all cases, proteins from *Spirulina platensis* have been investigated.\(^31,32\)

To study the gelation activity of alga extracts, the storage modulus \( G' \) [Pa] was measured during a defined heating–cooling profile. The storage modulus indicates the force required to deform certain material, and thus, it serves as a quantitative measure of the strength of the formed gels. The results are presented in Figure 5A for alga extracts prepared in this study and in Figure 5B comparing with data published for Rubisco from spinach and two commercial protein isolates (WPI and Egg White Protein EWP).\(^20\) At first, all fractions were prepared at 5% protein content. However, due to solubility constraints, only two fractions could be prepared at 10% protein content: CE and 0.45 \( \mu \text{m} \) P. WPI did not show any gel-like behavior at 5% or at 10% protein content, which was also observed by Martin et al.\(^20\)

During the heating phase (25 to 95 °C) the onset of gelation \( t_g \) marks the time at which \( G' \) starts increasing and reflects the thermal stability of the molecules in the sample. Rubisco, WPI, and EWP are stable at temperatures below 65 °C,\(^33\) 77 °C, and 84 °C\(^34\) respectively, and therefore long \( t_g \) are expected. For instance, \( t_g \)'s of 10, 12, 15 min are reported for gels formed with three different isolates containing 2.5–12.5% protein (Figure 5B).\(^20\)

For alga extracts containing 10% proteins (CE and 0.45 \( \mu \text{m} \) P), \( G' \) raised rapidly approximately 1 min after heating was initiated, which suggests low thermal stability. It appears that proteins denature quickly and readily form gels. On the contrary, for samples containing 5% proteins, the onset of gelation took place at longer times (2–15 min). This reflects the effect of concentration on the development of stable gels. In fact, gels are formed only above a critical concentration specific for each protein.\(^35\) For example, Rubisco can form gels at concentrations as low as 0.5%.\(^30\) On the contrary, Proteins from *Arthrospira platensis* could only form gels from 1.5 to 2.5% w/w and 12%.\(^32\)

Gels formed with 10% proteins show a strong increase in \( G' \) followed by a plateau at 95 °C (\( G'_{\infty} \)). A similar trend but with a moderate slope was observed for fractions containing 5% proteins (Figure 5A). This is possibly due to a lower availability of interacting molecules at 5%, which reduces the probabilities of forming new bonds upon heating. The values of \( G'_{\infty} \) for all samples are presented in Table 2. Interestingly, gels prepared with CE (10% protein) showed superior gel strength after the heating phase compared to 2.5% Rubisco (Figure 5B), 10% soy (\( G'_{\infty} \approx 400 \text{ Pa} \)), and 21.5% lupine (\( G'_{\infty} = 340 \text{ Pa} \)) protein isolates.\(^30\) WPI (12.5%) and EWP (10%) form substantially stronger gels, which may be due to the prevalence of noncovalent interactions which render them as rigid and brittle gels.\(^36\)

When the samples are cooled down to room temperature, a further increase in \( G' \) is observed for most fractions, except for the permeates of 300 and 10 kDa (Figure 5A). Martin et al.\(^20\) postulate that during this phase hydrophobic interactions and hydrogen bonds are primarily responsible for the development of a stronger gel network. Weak or lack of hydrophobic interactions in the permeates can indeed occur due to the hydrophilic nature of the membrane materials used during fractionation. When the temperature is sustained at 25 °C, a new plateau is reached (Figure 5) corresponding to \( G'_{\infty} \) (maximum gel strength). In Table 2 the values of \( G'_{\infty} \) are tabulated. Once more, gels prepared with CE or 0.45 \( \mu \text{m} \) at 10% protein registered the highest values, only comparable with 10% soy (\( G'_{\infty} = 1500 \text{ Pa} \)) and 17.5% pea (\( G'_{\infty} = 3100 \text{ Pa} \)) protein isolates.\(^30\) Gels formed with WPI and EWP greatly surpass the strength of the fractions investigated in this study (Figure 5B).

3.3. Functional Activity and Purity. We have shown that all alga extracts display a similar or superior functionality compared to the commercial protein isolate WPI. In addition, we observed that the retentate fractions presented better functionality compared to the crude fractions or permeates. This can be due to

i. Pigment–protein complexes, which in alga extracts have been found to stabilize emulsions and to form stable gels. The strong green color and a higher amount of total lipids (Table 1) indeed confirm that virtually all pigments are recovered in the retentate phase. In addition, due the hydrophilic nature of the membrane used in this research, the permeate fractions are expected to be depleted of hydrophobes. Under this condition,

| Table 2. Values of \( t_g \), \( G'_{\infty} \), and \( G'_{\max} \) and Corresponding Standard Deviations for Several Alga Fractions |
|---|---|---|---|
| sample | \( t_g \) [min] | \( G'_{\infty} \) [Pa] | \( G'_{\max} \) [Pa] |
| CE 10% | 1.2 ± 0.0 | 862.5 ± 133.6 | 2985.0 ± 473.8 |
| 0.45 \( \mu \text{m} \) P 10% | 1.5 ± 0.2 | 723.5 ± 125.2 | 2430.0 ± 424.3 |
| CE 5% | 14.5 ± 0.8 | 3.4 ± 0.6* | 4.5 ± 1.1 |
| 300 kDa R 5% | 2.0 ± 1.2 | 26.1 ± 4.2 | 145.5 ± 19.1 |
| 10 kDa R 5% | 8.8 ± 6.6 | 8.6 ± 0.6 | 60.0 ± 9.4 |

*Letters show significantly different means (p < 0.05) according to a t-test (samples at 10%) and Tukey’s HSD test (samples at 5%).
hydrophobic interactions in the permeates are limited, which in turn results in a poorer functional activity.

ii. The permeate fractions perform as soft particles or Pickering stabilizers. As presented in Figure 4B, the average particle size in the retentate fractions is significantly higher. Such particles correspond to large molecular aggregates or fragments of cell wall, membranes, and other cellular structures. Tenorio et al. studied the interfacial properties of thylakoid membrane fragments obtained from leaves and suggested as well that their functionality resembles that of Pickering stabilizers.

iii. Divalent cations. As mentioned before, the retentate fractions are likely to be enriched with cell fragments originated from the cell wall. The cell wall of Tetraselmis species have been found to contain approximately 4% of Ca\(^{2+}\) (DW). Divalent cations, like Ca\(^{2+}\), contribute to the development of bridges among charged sites of active molecules, therefore enhancing the strength of films around surfaces and networks within gels.

Besides the remarkable functional activity displayed by extracts obtained from green microalgae, their potential application as food ingredients is still constrained by the solubility, strong green color, risk of off-flavor, and economics. We have studied samples containing 0.1, 5, and 10% proteins, which are ranges commonly found in the literature. However, further exploration on how solubility is affected by pH, ionic strength, and concentration could provide more specific information on the possible applications in foods. For specific markets and products, the characteristic color and organoleptic properties of the retentate fractions or crude extract may impede their applicability. In terms of protein recovery, we have observed total yields of soluble proteins of about 12% after bead milling and 3–6% after filtration. Although the published yields of water-soluble proteins vary considerably (5–55%) depending on the algal strain and separation method, it is clear that the recovery of functional proteins from the insoluble phase is still the most important challenge.

The concept of functionality linked with purity and native conformation needs to be critically evaluated. Waghmare et al. observed excellent foam properties of the protein extracts obtained after a harsh process in which proteins were mostly denatured. Our research showed that excellent functionally can be obtained with crude samples, even after minimal separation steps (crude extract, Figure 1). This fraction therefore represents a more interesting option in terms of processing costs. In fact, technofunctional properties can be improved by exploiting other compounds present in algae, without the need of numerous purification steps. The presence of side products or impurities can actually enhance activity. Carbohydrates, pigments and lipids, ash and starch have been found to improve functional properties. Even whole biomass could be used as functional ingredients for some applications. This in turn will result in simpler and more compact downstream processing and, therefore, more cost competitive processes.

**Associated Content**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.8b01884.

Theoretical treatment of dynamic surface tension data for air–water and oil–water interfaces; fit to a mixed kinetic–adsorption model; strain sweep analysis of gels prepared with alga fractions (PDF)

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**Notes**

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