Strategies for Improving the Protein Yield in pH-Shift Processing of *Ulva lactuca* Linnaeus: Effects of Ulvan Lyases, pH-Exposure Time, and Temperature

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ABSTRACT: Globally, there is a need for novel vegetarian protein sources. We recently showed that the pH-shift process, using alkaline protein solubilization followed by isoelectric precipitation, is an efficient way to produce extracts with high protein concentrations from *Ulva lactuca* (>50% on a dry matter basis). However, the total protein yield was low, and to improve this, the effects of adding ulvan lyase, preincubating the seaweed homogenate at pH 8.5 and using different protein extraction temperatures (8 °C, RT, and 40 °C), were evaluated in this study. Addition of ulvan lyase reduced protein solubility but increased the precipitation. Incubation at pH 8.5, without ulvan lyase added, significantly increased both protein solubility and precipitation at 8 °C and RT. Temperature *per se* had no effect on protein solubility, while protein precipitation increased with decreasing temperature. Highest protein yield (29%) was achieved when keeping the process at 8 °C with a preincubation step at pH 8.5 for 1 h. By these process modifications, the yield was 3.2 times higher than achieved by the control process (9.2%).

KEYWORDS: Seaweed, *Ulva lactuca*, Ulvan lyase, Protein extraction, pH-shift, Protein yield

**INTRODUCTION**

In the light of an increased need for vegetarian proteins and a limited amount of cultivable land area and fresh water, seaweeds are, due to their relatively high protein content (up to 47% dry weight†), considered as an interesting food raw material. The green species *Ulva lactuca* is naturally found on the Swedish west coast and has shown good results during cultivation. Recently we showed‡ that the food grade pH-shift process, with protein solubilization at alkaline pHs and isoelectric precipitation aided by a freeze-thawing step, provide a concentrated protein extract (51% protein dw) from *Ulva*. However, the total achieved protein yield was very low (~6%), something that needs to be improved to obtain better utilization of the raw material. Several studies have shown that the extractability§—‖ and recovery¶ of seaweed proteins could be increased using enzymes acting on the cell wall. Recently, novel ulvan lyase, specific against the main polysaccharide ulvan in the cell wall of *Ulva*, was produced.§ The aim of this study was to find a strategy for improving the pH-shift process when applied to *Ulva* by evaluating the effect of adding ulvan lyase into the process. To accomplish this, an extra incubation step at pH 8.5 was required, and the effect of this step *per se* at different temperatures was also investigated.

**METHOD**

**Chemicals.** Sodium hydroxide was purchased from Scharlau (Barcelona, Spain), copper sulfate from Fluka (Buchs, Switzerland), and hydrochloric acid from Acros (Gothenburg, Sweden). Folin-Ciocalteu phenol, sodium carbonate, sodium dodecyl sulfate, and sodium tartarate were purchased from Sigma-Aldrich (Stockholm, Sweden).

**Seaweed.** *Ulva* was grown in cultivation tanks (90 L) under a neutral light cycle (16 h daylight, 8 h darkness) at a light intensity of 140 μE m⁻² s⁻¹. The light source was an INDY66 LED 60 W 4000 K 6000 lm. The seaweeds continuously received seawater that was passed through 1 μm filters. No additional medium or chemicals were added to the water. The natural seawater used in the flow-through system was pumped in from the bay outside the Tjärnö Marine Laboratory (58°52′36.4″ N, 11°6′42.84″ E). Thus, the salinity and temperature fluctuated depending on the prevailing weather and seasonal conditions. After harvest, the biomass was oven-dried (MK ASZ 25, Maurer, Domaszék, Hungary) at 40 °C for 24 h, stored at room temperature (RT), and ground using a coffee grinder prior to use.

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Table 1. Process Settings Evaluated in pH-shift Process of Ulva

<table>
<thead>
<tr>
<th>Sample name</th>
<th>A, control</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
<th>H</th>
<th>I</th>
<th>J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basic process temperature (°C)</td>
<td>8</td>
<td>8</td>
<td>RT</td>
<td>RT</td>
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<td>RT</td>
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<tr>
<td>1. Incubation at pH 8.5</td>
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<td>Time (min)</td>
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<tr>
<td>Temp (°C)</td>
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<tr>
<td>Ulvan lyase</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>2. pH 12 (min)</td>
<td>20</td>
<td>20</td>
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<td>80</td>
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<tr>
<td>3. Incubation at pH 8.5</td>
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<td>Temp (°C)</td>
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<td>RT</td>
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<tr>
<td>Ulvan lyase</td>
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<td>–</td>
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<td>–</td>
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</tbody>
</table>

*Plus (+) denotes that the given treatment was performed, minus (−) that it was not.*

Production of Enzymes. Ulvan lyase of *Formosa agariphila* (gene CDF79931) was cloned into the pET28a(+) expression vector and overexpressed in *Escherichia coli* BL21(DE3) and purified (a two-step procedure involving immobilized metal affinity and size-exclusion chromatography) according to Konasani et al. and kept at 4 °C until further use.

Extraction of Proteins Using the pH-shift Process. The basic pH-shift process protocol used here originated from the earlier published procedure performed at 8 °C. Here, 2 g of oven-dried *Ulva* was homogenized using an Ultra Turrax T18 basic (IKA, Germany) for 2 min at 18,000 rpm min⁻¹ in 55 volumes of water (which corresponds to 6 volumes of water based on the seaweed wet weight) followed by stirring for 1 h using a magnetic stirrer to allow for osmotic shock. Thereafter, the pH was adjusted to 12 using NaOH and incubated for 20 min, followed by centrifugation at 8000g for 10 min. The supernatant was recovered, the pH adjusted to 2 using HCl, and the sample frozen overnight at −20 °C. After thawing in cold water, a second centrifugation was done at 8000g for 10 min, and the pellet was recovered as our protein extract. Samples were withdrawn from the homogenate (hom) and supernatants 1 and 2 (sup1 and sup 2) for protein analysis. The protein solubility, protein precipitation, and total protein yield were calculated using recorded volumes (vol) of fractions according to eqs 1–3

Solubility = 100 × \(\frac{\text{conc sup 1}}{\text{conc hom}}\)  

Precipitation = 100 × \(1 - \frac{\text{conc sup 2}}{\text{conc sup 1}}\)  

Total yield = 100 × \(\frac{\text{conc sup 1} \times \text{vol sup 1}}{\text{conc hom} \times \text{vol hom}}\) \(\times \left(1 - \frac{\text{conc sup 2} \times \text{vol sup 2}}{\text{conc sup 1} \times \text{vol sup 1}}\right)\)

To improve process output, alternation of several factors were evaluated resulting in a total of 10 conditions of the process (Table 1 and Figure 1). Enzymes were added between the osmotic shock and the pH adjustment to 12. As ways to maximize the enzyme activity, we varied the temperature (RT and 40 °C) and added an extra incubation step for 60 min at the pH maxima of the enzymes, 8.5, before adjusting the pH to 12 (process versions E and J). The effect from applying this step alone at 8 °C, RT, and 40 °C was also investigated (B, D, I), as well as the effect of running the whole original process at RT (C) instead of at 8 °C (A), which was the control. Further, we investigated the effect of adding the enzymes after the first centrifugation step (H) and also the effect of a prolonged time for protein solubilization (F) at pH 12 (80 instead of 20 min).

Total Proteins. Total proteins in biomass, homogenate, and supernatant 1 and 2 were determined according to the method of Lowry et al. modified by Markwell et al. as earlier described in Harrysson et al.2

Sampling and Statistics. All different combinations in Table 1 were performed twice, and data are presented as mean values ± (max value − min value)/2. To determine if there were significant differences between data from the different treatments, *t* tests were carried out using R.11

RESULTS AND DISCUSSION

The protein content in crude *Ulva* was 12.8 ± 1.5% based on the dry weight, i.e., within the range of earlier published protein levels of *Ulva lactuca*.1,2,12,13

Adding enzymes after the osmotic shock step, followed by incubation for 60 min at pH 8.5, decreased the protein solubility at pH 12 both at RT (E) and at 40 °C (J), with 24.2 and 17.6 percentage points, respectively, compared to when no enzymes were added (D and I) (Figure 2). The average protein solubility achieved when using enzyme (E and J),
The solubility of Ulva proteins in water increases from ∼35% at the native pH (4.8) to ∼50% at pH 8–9. This indicates that solubilization of the proteins starts slightly before reaching pH 12. Several other studies, e.g., Hardney and FitzGerald and Veide Vilg and Undeland, have also shown that seaweed protein solubility increases with increasing pH. When running the process at RT, 60 min incubation at pH 8.5 followed by 20 min incubation at pH 12 (D) even increased the solubility more compared to just prolonging the incubation at pH 12 from 20 to 80 min (F). It is likely that some of the Ulva proteins that require a lower pH to solubilize then aids the solubilization of other more alkali-soluble proteins.

Incubation for 60 min at pH 8.5 also affected the protein precipitation positively, both at 8 °C (B) and RT (D). When studying the effect of this step alone, regardless of temperature, the average precipitation of process versions B and D (46.4%) was significantly (p = 0.017) higher than the average protein precipitation without incubation at pH 8.5 (A and C, 22.3%). However, incubation at 8 °C gave slightly higher precipitation and also a firmer pellet which was easier to recover, providing less variation in the results. Altogether, this resulted in version B, i.e., processing at 8 °C with 60 min incubation at pH 8.5 before further solubilization at pH 12, giving the highest total protein yield, 29.0%, which is 3.2 times higher than the yield achieved with the control process (A) in this study giving 9.2% yield and earlier 6.4%. The second and third best yields were achieved for version H comprising enzyme addition after the first centrifugation (25.9%) and D, the same process as B but run at RT instead of at 8 °C (25.7%).

Recent attempts to extract proteins from Ulva with water, or weak NaCl, in combination with pulsed electric fields resulted in 2.9%–12% extractability. By using cellulase or pectinase, or a high shear homogenizer, >25%–30% and 39%, respectively, of the proteins were extracted. No attempts to precipitate the solubilized proteins were made in those studies. Hence, some of our methods extracted remarkably higher levels of Ulva proteins, up to 63.7% using version B. Wong and Cheung achieved a final protein yield of 36% from Ulva, when solubilizing the proteins at pH 12 together with 2-mercaptoethanol and using ammonium sulfate for precipitation, hence higher than we found. However, the drawback of using 2-mercaptoethanol is that it renders the process nonfood grade. Our ultimate goal was to produce a new seaweed food protein ingredient, which is why use of 2-mercaptoethanol was not an alternative.

It is concluded that, to take advantage of ulvan lyase during pH-shift processing of Ulva, they should be added after the proteins have been solubilized at pH 12. This strategy raised the protein yield from 9.2% to 25.9%. However, it is estimated that it is not cost effective to produce ulvan lyase for this purpose, particularly because the highest protein yield (29%) was achieved when keeping the temperature at 8 °C, and only applying an extra incubation step at pH 8.5 prior to further solubilization of the proteins at pH 12. Running the process
under cold conditions is also recommended to protect the sensitive seaweed fatty acids from oxidation. We earlier found that fatty acids, including those of the n-3 family, concentrate together with the proteins during pH-shift processing of Ulva.  

**REFERENCES**

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The authors declare no competing financial interest.

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