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Article

Isolation and Gelling Properties of Duckweed Protein Concentrate

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ABSTRACT: An isolation procedure for proteins from duckweed was optimized based on a previously developed method for protein isolation from sugar beet leaves. Optimization included the protocol for disrupting cells and protein recovery. With the optimized protocol, protein was isolated (protein yield 14.2%, RuBisCO yield 27%). The concentrate was off-white and contained 67.2% protein. The isolation procedure resulted in a large enrichment in RuBisCO (from 48% to 92%). Denaturation of duckweed protein concentrate was observed at 62 °C at pH 7, while heating at pH 4 did not show denaturation peaks. Solubility was good far from the iso-electric point and showed a minimum around pH 5. Gelling was better at pH 7 than at pH 4. At pH 7, duckweed gels were much stronger than soy and only slightly weaker compared to egg white protein, while at pH 4 duckweed gel strength was similar to soy and lower than egg white.

KEYWORDS: *plant-based protein, technical functionality protein concentrates, gelation, protein isolation, novel protein sources, RuBisCO, biorefinery, duckweed*

INTRODUCTION

With the growing world population, there is the need to shift to a more sustainable consumption pattern. Sustainability in protein production is most commonly described by its footprint, taking into account greenhouse gas emission, water use, and arable land needed for cultivation. Generally speaking, plant proteins are more sustainable compared to animal protein. Some plant-based proteins have a somewhat imbalance in amino acid composition for optimal human nutrition. Cereals, seeds, and nuts are often low in lysine while legumes have an under representation of the sulfur amino acids, cysteine, and methionine.[1] A high (>95%) digestibility is usually observed with animal proteins (egg, milk, meat) and purified plant protein while a lower digestibility is observed with some less purified plant proteins potentially due to plant cell walls, antinutritional factors, or food processing and heat treatment.[2]

Proteins are used as ingredients in many food products, often to perform a functional role such as gelling, foaming, or emulsifying. A number of plant protein concentrates are commercially available, and these are increasingly used in the food industry as gelling or emulsifying agents, for example, in meat replacers, sauces, or high protein drinks. The most applied plant protein concentrates are derived from soy and wheat. In both cases, crop cultivation has a long history, and protein concentrate production has been optimized over the past decades. In the ongoing protein transition, other alternatives for animal protein than soy and wheat are gaining interest. Diversification of the limited set of currently available proteins is advantageous for multiple reasons, including (1) increase of biodiversity,[3] (2) a more diverse diet,[4] (3) increase the use of local crops (in multiple countries),[5] and (4) increase of the use of more side streams as food ingredients.[6−8] Furthermore, diversification also widens the possible applications of proteins in products, as every protein has its own unique properties. Often mentioned examples of upcoming protein sources are pea, fava bean, and other pulses, for which the protein isolation procedure is similar to that for soy. The most abundant protein on earth, RuBisCO, is currently not isolated commercially. RuBisCO is the protein that plays an important role in carbon fixation in all green plants. It is the main constituent of the more generally termed "leaf protein". In the 1970s and 1980s, RuBisCO isolation was investigated[9,10] and the investigation continues today[11] on multiple sources including duckweed.[12] Upscaling of RuBisCO isolation using alfalfa was investigated as well,[13,14] but no widespread commercialization followed.

Whereas some of the challenges to isolate plant proteins are similar to that of protein extraction from pulses, such as the dependence of the functional properties of the concentrate on the isolation procedure, a number of the challenges are unique to green leaf protein sources. Previous studies have investigated techniques to isolate protein from green leaves including spinach and sugar beet leaves.[15−17] The resilience of the leaf matrix makes it challenging to obtain a functional, white concentrate with little to no off-flavours. Oxidation

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products of lipids result in off-flavor, while chlorophyll remnants result in an off-color.

Most isolation procedures contain the following steps: first, mechanical plant cell disruption; second, precipitation by heat, pH, or a combination of both; third, purification.[14,18−20] Using these three steps pigments, fibers and antinutritional factors are eliminated from the mixture,[21] although a balance always needs to be found between removal of unwanted compounds and damaging the protein.

In the past years, our laboratory has been investigating isolation of proteins and protein functionality from duckweed (*Lemna gibba*). Duckweed has a high yield of biomass (and protein) per hectare in comparison with other sources. Under optimal conditions, duckweed could yield 10−18 tonnes protein/ha/year, compared to 0.6−1.2 tonnes protein/ha/year for soy in Europe.[22] These results are in line with growth in a nonheated greenhouse in a temperate maritime climate, as can be found in The Netherlands, and yields about 18 tonnes protein/ha/year.

For an alternative protein (such as protein from duckweed) to be a good replacement for animal protein currently consumed in the diet (such as egg or meat), several factors are of importance, including sustainability, nutritional value, functional properties such as emulsifying or gelling and organoleptic properties such as flavor and mouthfeel.

In this article, we describe the optimization of mild isolation of duckweed protein using a number of isolation procedures. The nutritional quality and technical functionality of the concentrate obtained with the final procedure is evaluated using composition and gelling studies, respectively.

MATERIALS AND METHODS

Materials. As reference proteins, the soy protein Supro500 (Solae, 92% protein on dry matter) and chicken egg white powder high gelling (Bouwhuis Enthoven, 92% protein on dry matter) were used.

Duckweed Cultivation. *Lemna gibba* (accession G3), furthermore referred to as duckweed, was produced in a greenhouse at Wageningen Plant Research with natural light together with artificial light for a maximum period of 18 h light a day and temperature between of 20 and 30 °C. From a sterily grown duckweed strain that was stored via biobanking, plants were taken each year to start up a small scale culture, from which lager trays could be "inoculated". Plants were grown in 10 L trays stationary on half concentrated Hoagland solution[23] in tap water without UV filters, water aeration, or water circulation.

Every 2 weeks the medium was fully refreshed, and plants were each week divided over an appropriate number of 10 L trays such that the plants at the start of the new tray were just covering the complete water surface. Over a period of 8 weeks a small amount of *in vitro* grown biobanked material was multiplied into 15 kg of fresh material.

Measurement Duckweed Composition. *Nutritional Composition.* Freeze-dried *Lemna gibba* (duckweed) plant material (n = 3) was analyzed for several main components of the nutritional composition at NutriControl[46] using standard analytic methods after grinding of the material. Crude protein level was analyzed using a standard and validated Dumas nitrogen analysis method (NEN-EN-ISO 14891, ANAL-10531). A conversion factor of 5.8 was used, based on previous analysis in our lab of RuBisCO from spinach, and also was used for sugar beet leaf RuBisCO.[17] Also, other nutritional components such as crude fat (by acid hydrolysation, ANAL-10497), ash (heating at 550 °C and using gravimetry, ANAL-10028 Q), carbohydrates (determined after hydrolysis by HPLC-PAD, ANAL-10204 Q), starch (amyloglucosidase, AGS, method, ANAL-10030 Q), and dietary fibers (in accordance to AOAC 991.43, ANAL-10436 Q) were all analyzed using standard and validated analysis methods. Water content was determined by weighing five duckweed samples before and after oven drying for 24 h at 105 °C.

Amino Acid Composition. Amino acid composition was analyzed according to the Waters Co. AccQ-Tag Ultra Derivatization manufacturers protocol with slight modifications.[47] Derivatization was performed as described in the protocol of the derivatization kit. In short, protein samples were dissolved in 200 μL of 4 M methanesulfonic acid containing 0.1% tryptamine, flushed with N_2 gas for 1 min, and hydrolyzed at 121 °C for 4 h in the dark. After hydrolysis, the sample was neutralized by adding 200 μL of 4 M NaOH, Norvalin was added as the internal standard and samples were diluted in the correct range before measurement. Similarly, standard solutions for all amino acids were prepared and analyzed. The AccQ-tag Ultra method originally designed for UPLC was adapted for use on an Acquity ARC UHPLC. An Xbridge BEH C18 2.5 μm 3.0 × 150 mm Column XP (Waters Corporation; Milford, MA, U.S.A.) at 55 °C was used in combination with the eluents A and B from the AccQ-Tag Ultra Derivatization kit. Injections of 1 μL were eluted at a flow rate of 0.971 mL/min using a 200 μL gradient composition (99.9% A at t = 1.2 min to 10% A/90% B at t = 21 min) for 21 min followed by 8 min 99.9% A eluents. For detection, a Waters Co. 2998 PDA detector equipped with a microbore flow cell was used and results were analyzed using the Waters Co. Empower software.

Proteomic Analysis. Freeze-dried duckweed plant material and freeze-dried extracted protein from the same source were subjected to proteomics analysis. For this 500 mg plant and extracted protein, material was ground of which 10 mg was suspended in 1 mL of 2% SDS/20 mM DTT. Suspensions were sonicated for 10 min followed by incubation at 60 °C for 30 min. From each suspension, 50 μg of protein, according to prior Dumas analysis, was used for trypsin (1:50) digestion after alkylation with 50 mM iodo-acetamide, according to the S-Trap Micro Spin Colum Digestion Protocol from ProtiFi (Huntington NY, U.S.A.).[48] After digestion, peptides were eluted with 50% acetonitrile and 0.1% formic acid. Eluates were dried by SpeedVac and subsequently dissolved in 40 μL of 2% acetonitrile and 0.1% formic acid. Peptide eluates were injected onto a nanoEASY LCII UPLC (ThermoScientific, Massachusetts, U.S.A.) and trapped onto a PepSep C18 2 cm × 100 μm trap column. Using a 60 min gradient from 6% to 20% to 30% and finally to 85% acetonitrile in 0.1% formic acid, peptides were separated on an analytical C18 column, 8 cm × 75 μm, 1.8 μm particle size at room temperature at a flow rate of 300 nL per minute. Column effluent was online connected to a Qexactive^{Plus} using a nanoFlex electrospray with a PepSep spray capillary of 10 μm ID. MS acquisition was performed using a DDA method with alternating MS1 scan at resolution 70 000 profile mode, AGC target 3e6, max IT 50 ms, scan range 400−1500 m/z, and subsequently 10 MS2 scans centroid mode, resolution 17 500, AGC target 5e4, max IT 100 ms with isolation window 1.6 m/z at NCE = 28 on with preferred peptide match ions of charges 2, 3, or 4 and a dynamic exclusion window of 30 s.

Peptide identification and quantitation were performed by processing the LC-MSMS data with MaxQuant[49] using a protein sequence database from UniProt selected for the taxonomy: 4469 being the genus *Lemna*, containing 415 protein entries, plus a database of frequent contaminant proteins (e.g., trypsin, keratins, BSA, and so forth).

Identified proteins were subsequently grouped in families based on enzyme/protein function and based on the label-free quantification iBAQ value the percentages of these categories (calculated versus total iBAQ per sample) were visualized. The protein composition of the starting plant material could be compared in this way with the composition of the extracted "duckweed protein".

Step 1 of Protein Isolation: Mechanical Treatment of Duckweed. The first step in protein isolation is mechanical treatment to open the cells and allow proteins to be solubilized. Two types of mechanical treatment were compared using a blender and a slow juicer.

Blender Treatment. For optimization of the isolation protocol, a duckweed sample was used that was stored overnight at 4 °C. The final protocol used fresh duckweed. Duckweed (145 g wet weight)

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was mixed with demineralized water (861 g). Two types of blenders were used: Vitamix and Thermomix. Two blender settings and blending times were used: low 2 min and high 1 min. The resulting mixture was centrifuged at 16 500×*g* for 15 min (Sorval Lynx) to remove the solids. The supernatant was decanted and the protein was recovered via coagulation or precipitation.

Slow Juicer. For optimization of the isolation protocol, a duckweed sample was used that was stored overnight at 4 °C. The final protocol used fresh duckweed. The duckweed was pressed with a slow juicer (Angel Juicer 7500), creating a juice fraction and a solids fraction. The juice fraction was used for the production of a protein concentrate product. Two routes were followed to isolate protein: precipitation and coagulation.

Step 2 of Protein Isolation: Protein Isolation from Juice. In the second step of the protein isolation, the juice fraction was further treated to yield a protein isolate, using either heat treatment, isoelectric point precipitation or a mild heat treatment followed by filtration.

Heat Treatment. The juice fraction was heated to 70 °C and kept at this temperature for 1 h to denature and precipitate the protein. Afterward the material was centrifuged at 16500×*g* for 15 min (Sorval Lynx). The supernatant was decanted and the pellet was collected.

Isoelectric Point Precipitation. The isoelectric precipitation protocol was adapted from Derksen et al.[24] The juice fraction pH was set to 4.2 with HCl. After 60 min, the material was centrifuged at 16 500×*g* for 15 min (Sorval Lynx). The supernatant was decanted, and the pellet was collected.

Mild Heat Treatment Followed by Filtration. A protein production trial was designed for the processing of 10 L of duckweed juice, requiring 12.5−15 kg of fresh duckweed. The duckweed juice was used fresh and was mixed with a 20 wt % sodium metabisulphite solution (pH 5.9) to a final concentration of 1 wt % sodium metabisulphite in the juice. The pH of the juice was set to 6.0 with HCl and the juice was subsequently heated in a jacketed, stirred metal container to 52 °C, and kept at this temperature for 33 min. Afterward the juice was quickly cooled down to less than 21.5 °C with tap water in the jacketed vessel. The juice was centrifuged in 1 L buckets (Sorval Lynx 6000) at 17000×*g* for 45 min. The supernatant was decanted and collected for filtration.

For optimization of decolorization, the pH, time, and temperature of the process were varied.

The first filtration step was cross-flow microfiltration (Sartorius Sartojet cross-flow system with 2 Sartocon slice cassettes (Hydrosart regenerated cellulose, 0.45 μm pore size), adding up to 0.2 m^2 membrane area)) to remove residual green material as well as most of the microbes. The second step was ultrafiltration (Sartorius Sartojet cross-flow system with three Sartocon slice cassettes (Hydrosart regenerated cellulose 100 kDa cutoff, adding up to 0.3 m^2 membrane area) in which water with salts and phenols were removed. The third and last step was diafiltration with demineralized water (Sartorius Sartojet cross-flow system with three Sartocon slice cassettes (Hydrosart regenerated cellulose 100 kDa cutoff, adding up to 0.3 m^2 membrane area)) in which salts and phenols were washed out until a final conductivity in the permeate of <0.2 mS. The final concentrated product was freeze-dried.

Functional Properties. All functional properties (solubility, denaturation temperature, and gelling properties) that are reported are performed on the last batch of the duckweed protein extract from 2020.

Solubility. To evaluate the impact of pH on the solubility profiles, the duckweed protein product was suspended in demineralized water at a concentration of 13 wt %, and the pH was set at 8.5 using 1 M NaOH. The pH of the protein solution was decreased stepwise from 8.0 to 3.0 using 1 M HCl, and 1 mL samples were taken for analysis of the solubilized protein.

Samples were then centrifuged at 15 000×*g* for 10 min and the protein content of the supernatant was measured in triplicate colorimetrically after a 10× dilution by using the Bradford method.[25]

Denaturation Temperature (Differential Scanning Calorimetry, DSC). Thermal analysis was performed with a TA Instruments type Q200 (Delaware, U.S.A.) modulated differential scanning calorimeter. Samples (25−30 mg, containing 10% or 12.5% protein) were weighed in high volume stainless steel cups, after which these were hermetically sealed. After an equilibration step of 5 min at −40 °C, the temperature scan was performed in linear mode from −40 to 160 °C with a rate of 5 °C/min. During this scan the heat flow was measured. The onset of protein denaturation (T_{onset}) and peak temperature (T_{peak}) were determined using the analysis tool available in the Universal Analysis software (TA Instruments). Measurements were performed in duplicate, and results are presented as mean values.

Gelling Behavior (Dynamic Mechanical Thermal Analysis, DMTA). Gelation kinetics were studied as a function of time using a stress-controlled Discovery HR-2 rheometer TA Instruments (Delaware, U.S.A.). A concentric cylinder system with a bob diameter of 14 mm was used. A temperature range was applied in which a stabilization of 5 min at 25 °C was followed by a temperature increase from 25 to 95 °C with a heating rate of 5 °C/min. It was kept at this temperature for 1 h (while measuring G' and G'' using a strain 10^{-3}, frequency 1 Hz) before cooling back to 25 °C. The applied strain was within a linear region. G', G'', and tan δ were measured during both steps as a function of time. At the end of the measurement a strain sweep was performed from 0.001 to 1 (25 °C). Samples were covered with a thin layer of paraffin oil to prevent evaporation. Measurements were performed in duplicate.

RESULTS AND DISCUSSION

Composition of Duckweed. Cultivated duckweed Lemna spp has a dry matter content of 6.3%. Several important nutritional components of the plant are shown in Table 1.

Table 1. Several Important Nutritional Components of Lemna spp

component	amount (g/100 g)	standard deviation (g/100 g)
crude protein (Dumas, N = 5.8) on DW	33.6	0.9
crude fat (by acid hydrolysation) on DW	3.4	0.2
crude ash (550 °C) on DW	18.0	0.4
total carbohydrates (HPLC) on DW	3.3	0.2
of which sugars	1.4	0.2
of which starch	1.9	0.1
dietary fiber on DW	25.5	0.7
moisture (on fresh weight)	93.7	0.9

The protein content of the material is about 34% on a dry weight basis, which was similar to other duckweed varieties reported in literature[12] and higher compared to other leaf sources used for protein isolation, such as spinach (protein content 28% on dry basis) and lettuce (25% protein content on dry basis),[26] and significantly higher than the protein content in, for example, sugar beet leaves, which is 18%.[27] Using proteomics analysis, we could identify and (semi)-quantify the individual main abundant proteins present in duckweed. The proteins present in the starting material consist of almost 50% of RuBisCO (Figure 1, left-hand side). In other sources, such as sugar beet leaf, RuBisCO is soluble and highly functional.[17,28] Other main proteins found in the duckweed starting material are photosystem proteins, other enzymes (such as ATP synthase and glutamine synthase), and ribosomal proteins.

Isolation of Protein from Duckweed. Isolation of proteins is generally done by first dissolving and subsequently precipitating the proteins from the solution. The specificity of

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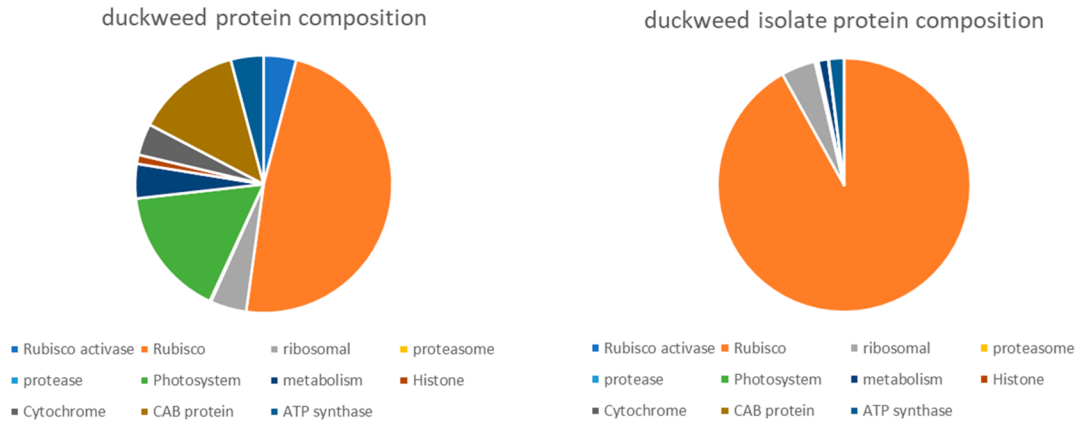

Figure 1. Protein composition of duckweed (left) and duckweed protein concentrate (right).

both processes determines purity and yield of the concentrate. The process can be divided in three steps: (1) pretreatment of the material, (2) cell disruption and solubilization, and (3) recovery of the protein. In this article, we explore optimization of these steps.

Screening of Protocols. In order to increase shelf life for the experiments and to induce some cell disruption, frozen and cool storage were compared. In a first set of experiments (data not shown), we found that although freezing increases shelf life and with that increases processing flexibility, while it also decreases the green color, the process is disadvantageous because of protein loss, bitter taste and higher energy use. Therefore, freezing was disregarded as a processing route.

For cell disruption, a comparison was made between blender and slow juicer treatments, while also an alkaline treatment (solubilizing at 0.1 M NaOH) without mechanical pretreatment was tested (Figure 2). The alkaline treatment resulted in

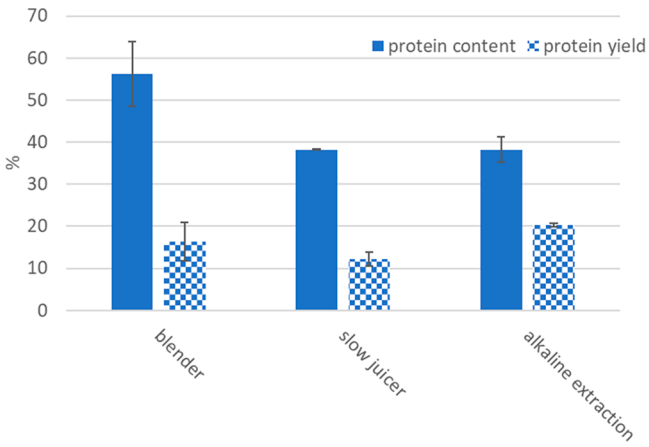

Figure 2. Protein yield and protein content of extracts made with different processing.

nonfunctional protein and was therefore not pursued further. Slow juicer and blender treatments each had their pros and cons. For an optimal protocol with the blender, dilution of the wet duckweed material (~5% dry weight) to a dry weight percentage of 0.5−0.7% was necessary, while the slow juicer was used on the (wet) duckweed as such, yielding a large advantage of the slow juicer in terms of water usage and drying costs. Also, the slow juicer treatment could be scaled up more easily compared to the blender treatment. However, treatment with the slow juicer yielded a larger amount of cell wall debris in the juice fraction compared to the soluble fraction after using a blender (shown in the low purity of the extract). This disadvantage was countered by adding a step in the process where with mild coagulation chlorophyll and cell wall debris are removed, while the protein stays in solution.[29] With that method in place, the slow juicer was chosen to disrupt the cell walls of the material for subsequent experimental work.

In the final step of the process, the recovery of the protein, three protocols were compared: (1) heat coagulation, (2) isoelectric point precipitation, and (3) filtration steps. In the heat coagulation, the soluble fraction of the protein was heated for an hour at 70 °C in a water bath, which was expected to result in denaturation and precipitation of the protein that could then be collected in the pellet. In the final protein extract, both a very low yield (6%) and a low purity (30%) were observed, which is the reason this option was not further used. Isoelectric point precipitation was performed by decreasing the pH of the solution to 4.2 using hydrochloric acid. The alternative filtration consisted of three steps, starting with microfiltration, followed by ultrafiltration, and finally diafiltration. In our experience in protein isolation, the absence of a precipitation step generally yields a protein that is easier to dissolve. A higher solubility is often a prerequisite for other technical functional properties, such as foaming, emulsifying, or gelling. The lower solubility after isoelectric point precipitation is confirmed by bringing the concentrate obtained by filtration to the isoelectric point, which yielded an insoluble protein even after increasing pH to 7.

Mild Coagulation to Remove Chlorophyll and Cell Wall Debris. In order to use filtration steps to recover the protein, first suspended cell wall material and green color have to be removed, which were performed using a mild heat treatment. Since heat and pH may also cause heat denaturation and coagulation of the soluble protein fraction (largely consisting of RuBisCO), an optimization is needed to ensure desired removal of as much suspended solids as possible, while leaving the soluble protein fraction mostly intact. A range of temperature and pH values were chosen based on previous experience to find optimal conditions. Temperature was set to 50, 52, or 54 °C, pH to 6.0, 6.25, or 6.5, for a time of 20, 30, 40, or 60 min (including heating up time of 7 min). In the Supporting Information, the first optimization step, comparing pH 6 and pH 6.5 at the different times and temperatures on frozen duckweed is shown. For all conditions, pH 6 resulted in a higher protein content in the liquid fraction compared to pH

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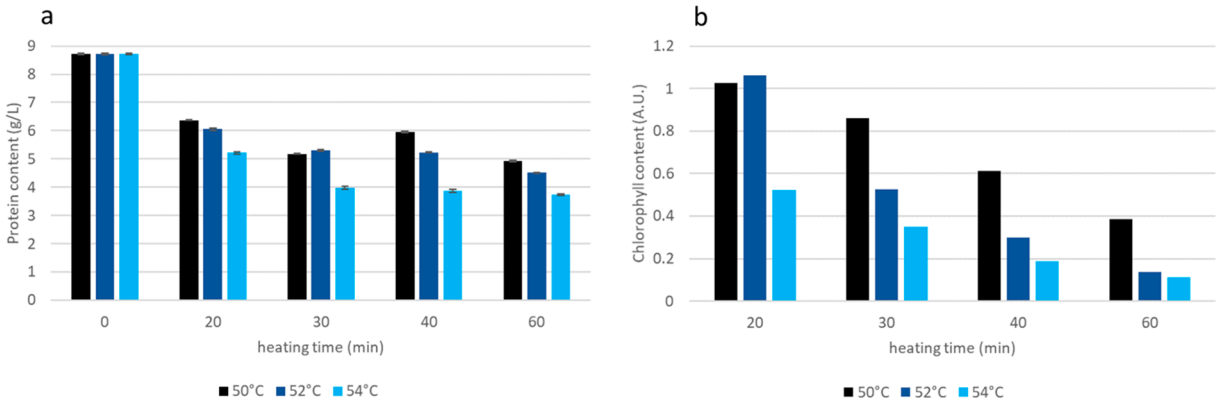

Figure 3. Effect of heat treatment at pH 6.0 on (a) protein content and (b) chlorophyll content in a duckweed extract.

Table 2. Yield, Purity, and Color of the Different Extractions

extraction	protein yield (% of total protein)	RubisCO yield (% total RubisCO)	protein purity *(% dry matter)*	color
A (= 2018)	5.6	not analyzed	56.5	greenish
B (= 2019)	3.8	not analyzed	64.6	off-white
C (= 2020)	14.2	27	67.2	off-white

Table 3. Protein Isolation from Leaf Materials

source	protein yield (% of total protein)	protein purity (% dry matter)	characteristics	aim of isolation	reference
duckweed	45.6	67.8	partly reported: low functional	information on functional properties	Yu et al., 2011[12]
spinach	3	95	highly functional	isolation (labscale) of very pure protein	Martin et al., 2014[28]
sugar beet leaves	11	86.4	highly functional	scalable isolation of protein	Martin et al., 2019[17]
siris	6	37	not reported	nutritional purposes	Khan et al., 2018[31]
poplar leaves	7.8	60	not reported	nutritional purposes	Khan et al., 2014[32]

6.5 (Supporting Information, Figure S1 and S2). Therefore, the optimization on the fresh duckweed process was performed only at pH 6.0. Chlorophyll content reduction was, as expected, increasingly effective with longer duration and higher temperatures. Protein content decreased with heating, also in line with expectation, but for 50 and 52 °C there was little difference between treatments of 20, 30, and 40 min, while a further decrease was observed after a 60 min heating step. For the 54 °C treatment, a further decrease between 20 and 30 min treatment is observed, while also in this case the protein content is rather constant upon further treatment. A pronounced decrease in soluble protein content is observed when the duration of the treatment is increased. A balance needs to be found between minimizing the amount of chlorophyll and maximizing the amount of soluble protein. At a treatment time of 20−40 min, there is little difference in protein yield between the 50 and 52 °C treatment, while the chlorophyll content decreases significantly with higher temperatures and times (Figure 3b). Therefore, a treatment of 40 min or 33 min after the final temperature was reached, at 52 °C and pH 6.0, was chosen. A heat treatment at these conditions removes most of the chlorophyll, while most of the soluble protein remains soluble (40% reduction in soluble protein, compared to the untreated sample from 8.7 to 5.2 g/L).

To decrease phenol oxidation, a 20% sodium metabisulfite solution (pH 5.9) was added to the duckweed juice during the slow juicing process to a concentration of 1%. After treatment with the slow juicer, suspended cell wall material and part of the green color were removed with a mild coagulation step at pH 6 during 33 min at 52 °C. The coagulation step was followed by centrifugation and a series of filtration steps, starting with microfiltration, followed by ultrafiltration and finally diafiltration.

Composition of the Duckweed Extract. Protein yield and purity were analyzed for three different extraction runs (as reported in Table 2). The difference between the protein batches was relatively high due to variation in the starting material, as is generally seen for natural materials.[30] Furthermore, with increasing experience the protocol was also performed more smoothly.

In the final batch, a yield of 14.2% was achieved with a protein content of 67.2%. The color of the protein concentrate was off-white to light green (Table 2 and Figure S3 in the Supporting Information) The protein content is very similar to a duckweed extract mentioned in literature, but the yield in the literature reference was higher (46%).[12] The reference uses an isoelectric point separation, which may have resulted in a decrease in solubility and via that in the low reported functionality. Color was not reported. A brief comparison with some literature references is shown in Table 3. For a number of the leaf materials in the table, the isolation was optimized on protein yield and purity, while aspects such as color, solubility, or technical functional behavior were not mentioned.[31,32] In another article, a functional protein was isolated with a yield of 11% and a purity of 86% in the same range as the duckweed concentrate.[17] Duckweed yielded 34 g

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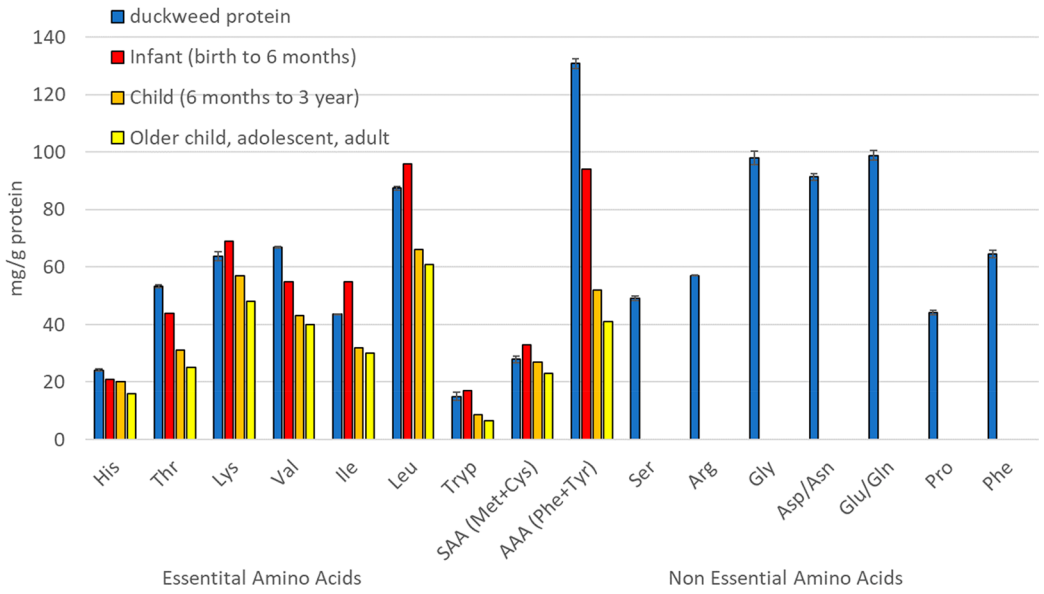

Figure 4. Essential amino acid composition of duckweed protein compared to WHO standards.

protein per kg dry material, whereas sugar beet leaf yielded less, with 23 g per kg dry material. That can be explained from the higher protein content of duckweed versus sugar beet leaf (34% vs 18% on a dry weight basis). In general, isolation of a highly pure and functional protein[28] entails a suboptimal yield.

Also, on the extracted protein sample proteomics analysis has been performed in order to determine the composition of the extracted protein fraction and to analyze if a purification or concentration of specific proteins has been established. From the proteomics analysis, it is clear that the isolation of the protein using our method results in a sample that is highly enriched in RuBisCO (see Figure 1, right-hand side). With RuBisCO from other sources reported to be soluble and highly functional,[17,28] this is both expected and favorable for the application of the extract. A large part of the other proteins in the duckweed is expected to be bound to the cell wall and thus be insoluble, explaining the large RuBisCO enrichment in the extract.

From literature, we know that RuBisCO is rich in essential amino acids.[33−35] Its amino acid composition has been reported to be comparable to that of soybean, meat, fish, and eggs,[36−40] making it an excellent source from a nutritional perspective. The amino acid composition of the current extract is in line with this information (Figure 4). The concentrate contains a balanced amino acid composition, close to the required optimal concentrations of essential amino acids as set by the FAO/WHO for infants (0−6 months). Concentrations of essential amino acids are even higher than those set for children (6−36 months) and older aged. Furthermore, the concentrate is high for arginine, glycine, glutamine, and proline which are conditionally essential in the human diet, meaning that these amino acids are essential for people that suffer from limited synthesis due to pathophysiological conditions. As several plant-based proteins are low in lysine, leucine, tryptophan, or sulfur-containing amino acids, this protein can be an interesting source as such or can be used in protein blends to develop products with optimal composition.

Use of Duckweed Protein Concentrate in Food Products. To use duckweed protein concentrate in food products, technical functionality, such as gelation and emulsification, is very important, depending on the application. In this article, we focus on gelation as a functional property, which is essential for structure and "bite" in products. RuBisCO concentrates from sugar beet leaf and spinach are known to have a lower gelation temperature and higher gel strength compared to egg white gels, and to be more brittle than egg white protein gels or whey protein gels. This combination of properties allows for unique applications for the plant-based RuBisCO protein.[17,28]

In order to be able to gel a protein, generally a high solubility is required. In most products, a gel is achieved using a heat-set gelation protocol. The gelation occurs by denaturing the protein which subsequently aggregates into a gel structure. This gelation protocol is only feasible if the heating step reaches a temperature above the denaturation temperature. Therefore, also denaturation of the protein is measured.

Solubility. The maximum solubility of the protein is 70% at a pH over 7. The solubility of protein in Figure 5 is given

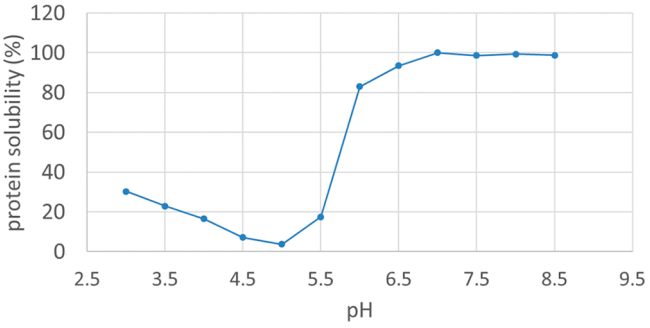

Figure 5. Solubility of duckweed protein concentrate, measured at a series of pH values.

relative to the maximum solubility. The protein shows a classical profile with a solubility decrease in the pH range of the isoelectric point. The isoelectric point as derived from the solubility curve is around 5, similar to that of spinach and sugar beet leaves.[17,41] Values of the isoelectric point are shown to vary between 5 and 7 for different species,[42] which shows the relatively large difference between this type of protein between

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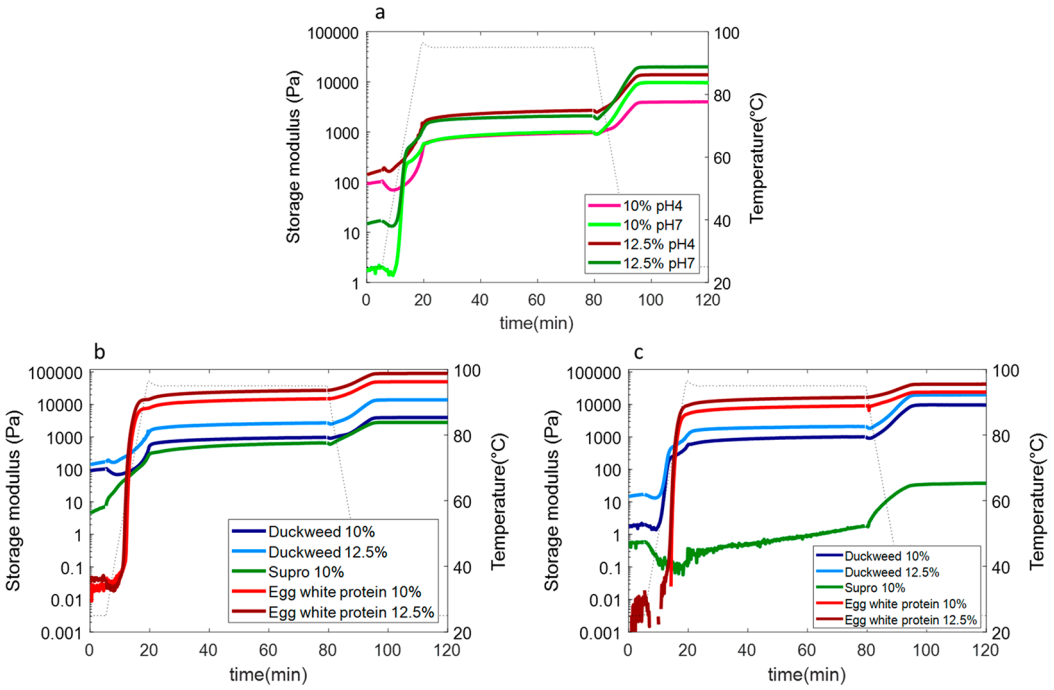

Figure 6. G' of protein concentrates at different pH values. (a) Two concentrations of duckweed protein; bottom: duckweed protein compared to soy protein and egg white protein at (b) pH 4 and (c) pH 7.

species. The data for the duckweed protein concentrate fall in this range. Maximum solubility is observed at alkaline pH. The maximum solubility was somewhat lower than described for RuBisCO from sugar beet leaves (~90%),[17] which may be the result of changes in the protein configuration that was induced during the isolation process.

Denaturation. Denaturation of the duckweed protein showed at pH 7 an onset around 54 °C (54.2 °C ± 0.5 °C) and a peak position at about 62 °C (61.0 °C ± 0.2 °C) (the graphs can be found in the Supporting Information, Figure S4). This denaturation temperature is somewhat lower than reported in other publications.[28,43,44] At pH 4, no peaks were observed, indicating that the pH treatment denatured the proteins. This result is in line with literature data of Lucerne RuBisCO.[44,45] Stability of RuBisCO protein is known to be different between sources, and apparently the RuBisCO variety in duckweed is less stable compared to that of some other crops. The lower denaturation temperature is in accordance with the lower solubility at alkaline pH (only 70% compared to the 90% reported for sugar beet leave RuBisCO),[17] which was hypothesized to be caused by a less stable RuBisCO protein. The lower denaturation temperature indicates lower stability, and thus more denaturation may have taken place during isolation despite the mild conditions chosen.

Gelling Behavior. The gelation of duckweed protein was investigated by measuring the storage modulus (G') as a function of time during heating, holding temperature at 95 °C, and cooling down, similar to the experiments described by Martin et al.[28] Protein concentrations of 10 and 12.5 wt % were compared at pH values of 4 and 7 (relevant conditions for food products). The viscosity of the starting material was much higher at pH 4, shown by a higher G' (Figure 6a). The high viscosity is in line with partial denaturation and aggregation of the material. During the heating step at 95 °C, the G' of the material at the two pH values is very similar, while the G' of material at pH 7 increases more upon cooling, yielding a slightly higher final gel strength. The difference between the gel strengths is relatively small, showing a possibly applicability of the protein over a large pH range.

The soy protein isolate Supro 500 and egg white protein were used as a reference (Figure 6b,c). Compared to the Supro soy protein, the duckweed protein yields similar gel strength at pH 4 and a far stronger gel at pH 7. Egg white protein, the golden standard in gelation, yields higher gel strength at both pH values. However, at pH 7 a 12.5% duckweed protein gel has almost the same gel strength as a 10% egg white protein gel, which is remarkably high. Interestingly enough, an increase in pH yields stronger gel for the RuBisCO, and a weaker gel for both soy and egg white protein. At pH 4, similar gel strength was obtained for the soy compared to the duckweed protein and the egg white gel is clearly much stronger, but at pH 7 the duckweed yielded a much stronger gel than soy and almost the same strength as egg white protein. These results show the high potential for the duckweed protein as an egg white replacement, especially at neutral pH.

All in all, this article shows how a sustainable but as yet not employed source can be used to produce a protein concentrate with excellent gelling functionality over a large pH range.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsfoodscitech.1c00009.

Additional figures (PDF)

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Notes

The authors declare no competing financial interest.

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