Supramolecular structure modification of RuBisCO from alfalfa during removal of chloroplastic materials

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ABSTRACT

The use of alfalfa as a dietary protein source is of great interest. Membrane filtration shows potential for removal of unwanted compounds in the purification of ribulose-1,5-biphosphate carboxylase/oxygenase (RuBisCO), but fouling remains a challenge. It was hypothesized that treatments, namely centrifugation, ultra centrifugation, and heat clearing, prior to membrane processing modify the supramolecular assembly of the proteins, facilitating its filtration. The pre-treatments led to different aggregate structures. In particular, heating at 50 °C decreased the aggregate sizes by ~500 folds relative to the raw juice, as measured by light scattering. The molar mass of RuBisCO decreased by ~180 kDa after treatment with heat. Similarly, supernatants after ultra centrifugation also showed smaller structures. Unlike the other treatments, heating led to an efficient transmission of RuBisCO during microfiltration. These results demonstrate for the first time, the importance of controlling the supramolecular structure of RuBisCO when processing proteins from alfalfa juice.

Industrial relevance: The demand for more sustainable protein sources is continuously growing. RuBisCO is of particular interest because of its favorable amino acid composition. However, improved purification strategies are needed to extract it from green biomass. The results of this study serve as a proof-of-concept for the application of an industrially relevant process, mild heat treatment in combination with microfiltration, for the purification of RuBisCO from alfalfa green biomass.

1. Introduction

The continual increase of world population has raised protein demand, and has challenged the sustainability of the existing food and protein production system. There is, therefore, an increasing need to investigate protein sources with less climate impact (Godfray et al., 2010; Henchion, Hayes, Mullen, Fenelon, & Tiwari, 2017). A potential source of such protein is the green biomass alfalfa (Medicago sativa L.), which is a perennial legume that can yield up to 1000–3000 kg crude protein per hectare (Solati et al., 2018). The alfalfa crop also possesses a low environmental impact for protein production as quantified using life cycle assessment, showing less global warming potential, ozone layer depletion, energy demand, eutrophication potential, acidification potential, and land use than other alternative plant protein sources, such as ryegrass and sugar beet leaves (Skunca, Romdhana, & Brouwers, 2021).

Furthermore, alfalfa has been determined safe for food applications by the European Food Safety Authority (EFSA), however, at this point, in a limited amount of 10 g/day when consumed as a concentrate (Bresson et al., 2009).

The purification of proteins from green leafy biomass, including alfalfa, is generally laborious due to the abundance of undesirable compounds, namely anti-nutrients, off-flavor compounds, phenols, and chlorophylls (Di Stefano, Agyei, Njoku, & Udenigwe, 2018). Various methods have been implemented for the purification of proteins from alfalfa, such as acid precipitation (Lamsal, Koegel, & Gunasekaran, 2007), heat fractionation (Fremery et al., 1973), ion exchange chromatography (D’Alvise, Lesueur-Lambert, Fertin, Dhusler, & Guillochon, 2000), microbial fermentation (Nissen, Lübeck, Møller, & Dalsgaard, 2022), and membrane filtration (Zhang, Grimi, Jaffrin, & Ding, 2015). Among these methods, the use of membrane technology, namely microfiltration (MF) and ultrafiltration (UF), is incentivizing in terms of
scalability, energy efficiency, and absence of harsh chemicals (Di Stefano et al., 2018; Zhang, Grimi, Jaffrin, Ding, & Tang, 2017). Some examples of the described use of membrane technology in the literature include separating soluble proteins from chlorophyll in algae (Saifi et al., 2017) and purifying sugar beet leaf protein in a demo plant associated with Cosun (Skunca et al., 2021). Furthermore, membrane processes are already extensively used in the dairy industry (Reig, Vecino, & Cortina, 2021), meaning that less investment is needed if the same technology can be applied for alfalfa.

The use of UF and MF is, however, challenging due to the need to control concentration polarization on membrane surface which causes the formation of a gel layer that can impair the viability of the filtration process by compromising the selectivity of the membrane pores, fluxes and form irreversible fouling. These problems have been highlighted in the past, in a study of microfiltration of green juice from alfalfa with 0.2 μm membranes (Eakin, Singh, Kohler, & Knuckles, 1978).

There is also limited information regarding supramolecular organization of the RuBisCO in solution and their changes during membrane filtration (MF/UF), leading to the limited understanding of the appropriate membrane pore sizes for fractionating it from undesirable components of the matrix. The major soluble protein of alfalfa is ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) (Hojilla-Evangelista, Selling, Hatfield, & Digman, 2017), which is known to exist in nature as a ~550 kDa hexadecamer of eight large (~55 kDa) and eight small subunits (~15 kDa) (Di Stefano et al., 2018). Furthermore, the average size distribution of RuBisCO, as determined by dynamic light scattering (DLS), was shown to be approximately 15 nm (Desai, Streeland, Wijffels, & Eppink, 2014). Therefore, it is possible to hypothesize that the majority of alfalfa proteins would permeate though 0.2 μm membrane pores, if RuBisCO exists as singular hexadecamers. However, Zhang et al. (2015) compared MF (nominal size: 0.2 μm) and UF (nominal molecular weight cut-off: 20 kDa) using three different filtration modules set-up, and concluded that due to the flux limitation and the severity of irreversible fouling, MF is preferred to UF for concentrating proteins in alfalfa juice. The concentration, as opposed to permeation, at nominal pore size of 0.2 μm indicates that either fouling has compromised the membranes or RuBisCO does not exist as singular hexadecamers in alfalfa juice, and might be instead present in larger supramolecular assemblies. Since membrane pore size governs the separation efficiency of the filtration process, it is therefore paramount to comprehend the supramolecular structure of the proteins in alfalfa juice and its potential changes during changes in the processing parameters in conditions with minimum fouling.

Based on the abovementioned scientific gap, we investigated the separation behavior of alfalfa protein extracts undergoing different pre-treatments on 1 μm MF membrane. The three different pre-treatment process, namely clarification, ultra centrifugation, and mild heat clarification, were expected to affect the separation behavior differently, as they will influence the aggregated state of the protein in solution. A clarification process with low speed centrifugation was tested, as it is a scalable method to remove insoluble components (i.e. fibers) (Amer, Juul, Møller, Møller, & Dalsgaard, 2021). Ultra centrifugation was used as it is an established laboratory-scale process for separating soluble RuBisCO from chloroplastic suspensions (Amer et al., 2021; Singer, Eggman, Campbell, & Wildman, 1952). In addition, a thermal treatment at 50 °C for 30 min was also tested as mild heating combined with clarification is deemed as one of the most efficient and scalable chlorophyll removal methods that has been used in a protein purification processes from alfalfa (Nynas, Newson, & Johansson, 2021). The investigation was carried out on alfalfa juice that were treated with sodium sulfite as the interaction between proteins and oxidized polyphenols has also been shown to affect protein structure (Kroll, Rawel, & Rohn, 2003). In particular, larger bands attributed to polyphenol and protein interactions have been observed in alfalfa protein concentrate purified with acetic precipitation, and were shown to be reduced with the addition of sodium sulfite (Tanambell, Møller, Corredig, & Dalsgaard, 2022).

To better test the membrane selectivity, the filtration was performed using a laboratory-scale, cross-flow filtration system, under vibrating conditions, so that the gel layer formation can be kept to a minimum, allowing the membrane pore size to govern the separation process with minimum interference. It was hypothesized that the pre-treatments (clarification, ultracentrifugation, or mild heating) would affect the size distribution of the protein aggregates, and therefore a better control of the upstream process would improve the fractionation of RuBisCO during downstream membrane filtration. This research will serve as a probe for future applications for purification of RuBisCO using MF as a fractionation method.

2. Materials and methods

2.1. Chemicals and materials

Chemicals used in this study were of analytical grade unless mentioned. Sodium sulfite, hydrochloric acid (HCl) 37%, sodium hydroxide, D-ribulose 1,5-bisphosphate carboxylase/oxygenase from spinach, bovine serum albumin (BSA), sodium dodecyl sulfate (SDS), Trizma® base, Trizma® HCl, sodium chloride, glycerol, bromophenol blue, PageRuler™ Plus Prestained Protein Ladder, phosphoric acid 85%, trichloroacetic acid (TCA), sodium azide, NaH2PO4, Na2HPO4, ammonium bicarbonate, iodoacetamide, α-cyano-4-hydroxycinnamic acid, POROS™50 R2 Perfusion Chromatography™ bulk media, formic acid, acetonitrile, and 1,4-dithioerythritol (DTE) were obtained from Merck (Darmstadt, Germany). Coomassie Brilliant Blue (CBB) G-250 and 18-wells Criterion TGX pre-cast gel (12%) were purchased from Serva (Heidelberg, Germany) and Bio-Rad Laboratories Inc. (Hercules, California, USA), respectively. NativePage™ 4–16% Bis-Tris Mini Protein Gel, NativePage™ Sample Prep Kit, and NativeMark™ Unstained Protein Standard were acquired from Thermo Fisher Scientific (Roskilde, Denmark). Ethylenediaminetetraacetic acid (EDTA) and 96% ethanol were purchased from Gerhardt Analytical Systems (Königswinter, Germany) and VWR International (Semborg, Denmark), respectively. Phenomenex™-Glass Fiber 28 mm syringe filters (1.2 μm) were purchased from Phenomenex® (Torrance, California, USA). Sequencing Grade Modified Trypsin were obtained from Promega (Madison, Wisconsin, USA).

MF modules with polypropylene housing (membrane area: 35 cm2) were purchased from SaniMembranes (Farum, Denmark). The modules were installed with hydrophilized polytetrafluoroethylene (PTFE) membranes with a nominal pore size of 1 μm (SANi; SaniMembranes, Farum, Denmark). Demineralized water (18.2 MΩ) was prepared in-house using the Milli-Q purification system from Millipore Corporation (Burlington, Massachusetts, USA).

2.1.1. Plant materials

The alfalfa plant materials were harvested using a MaksiGrass® (MaksiGrass, Denmark) from a field at Aarhus University Research Center, Foulum, Denmark, on 15 September 2021. Approximately 100 kg of the harvested biomass were immediately transported to the Department of Food Science, Aarhus, Denmark, and stored at −20 °C until further processing.

Abbreviations

<table>
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<tr>
<th>Abbreviations</th>
<th>Description</th>
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<tr>
<td>RJ</td>
<td>Raw Juice</td>
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<tr>
<td>CJ</td>
<td>Clarified Juice</td>
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<tr>
<td>UJ</td>
<td>Ultracentrifuged Juice</td>
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<td>HJC</td>
<td>Heat Clarified Juice</td>
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</table>
2.2. Sample preparation

2.2.1. Preparation of microfiltration feed materials

The alfalfa juice was prepared as previously described (Tanambell et al., 2022) with minor modifications. In brief, 330 g of frozen alfalfa stems and leaves was thawed at room temperature for about 30 min. The biomass was then soaked in 110 mL of 75 mM sodium sulfite. Both the screw press Angel® Juicer 8500S juicer (Angel®, Juicer, South Korea), resulting in final sulfite concentration of approximately 25 mM. The pH of the juice was adjusted to 6.00 ± 0.01 using 6 M HCl, which is the average native pH of alfalfa juice (Ajibola, 1984).

The juice was then subjected to three individual treatments (Fig. 1): clarification by low speed centrifugation, ultracentrifugation, and incubation at about 50 °C for 30 min (mild heat treatment). Clarification was conducted by removing the insoluble material through a 15 min centrifugation at 1700 ×g and room temperature using a Multifuge 3SR (Fischer Scientific®, Norway). For the ultracentrifugation treatment, removal of insoluble material was carried out first, by using the clarification treatment described above, followed by the removal of colloidal suspensions using an Optima L-80XP (Beckman Coulter Inc., California, USA) at 100,000 ×g and room temperature for 1 h. The mild heat treatment was adopted and modified from Nynás et al. (2021). In brief, the juice was heated in a 500 mL Duran borosilicate glass bottle (Schott AG, Germany) for approximately 30 min, using a SW23 shaking water bath (Julabo, Germany) at 50 °C and 50 rpm, resulting in a final temperature of 45 °C. The juice was then cooled down for approximately 20 min to room temperature using an ice bath, before being centrifuged at 1700 ×g and room temperature for 15 min. The supernatants of each of the three treatments were then subjected to MF and analyses as described in the following sections. While the untreated juice is referred to as raw juice (RJ), the clarified, ultracentrifuged, and mild heated centrifugal supernatants are subsequently referred to as CJ, UJ, and HJC, respectively.

2.2.2. Microfiltration

A vibrating, cross-flow Vibro-Lab35P system (SaniMembranes, Denmark) installed with 1 μm hydrophilized PTFE membrane was used for the MF. The selection of hydrophilized PTFE membrane was chosen per recommendation from the manufacturer, due to their modification, which renders the membrane more hydrophilic than polyvinylidene fluoride (PVDF). The MF process was performed at room temperature with a low transmembrane pressure of <0.05 bar. Vibration of the Vibro-Lab35P was driven by compressed air at a pressure of 6 bar. A volume of 100 mL centrifugal supernatant from the treatments described above was used as a feed material for the filtration process. The filtration process was carried out until approximately two volumetric concentration factor was achieved on the retentate side. Flux measurements and permeate collection were carried out at the beginning, middle (approaching 1.5 volumetric concentration factor), and end of the filtration process. In addition, the final retentate and the pooled permeate at the end of the filtration were collected for analysis. Samples were frozen at −20 °C immediately after collection. After each experiment, the membranes were cleaned using a clean-in-place (CIP) procedure provided by the manufacturer (SaniMembranes, Denmark). The extent of fouling was measured evaluating the change in flux with water prior to use and after the CIP of each experiment. Two separate batches were performed for each experiment, with the exception of the CJ, which was only performed once due to the irreversible fouling that formed on the membrane.

2.3. Analyses

2.3.1. Determination of TCA-precipitated protein and non-protein nitrogen

A DUMATHERM® (Gerhardt Analytical Systems, Germany) was used for determining the total Nitrogen content of the RJ, CJ, UJ, HJC samples, and the microfiltered fractions. Samples were combusted with 1.4 mL O₂/mg sample with an oxygen flow rate of 300 mL/min. The peak obtained from the measurement were then converted to total Nitrogen using an external calibration curve prepared from Trizma® base and EDTA.

A TCA-precipitation method adapted from Koontz (2014) was carried out to precipitate Nitrogen associated with protein. In brief, samples were diluted at a 1:1 ratio with cold 24% TCA, resulting in a final concentration of 12% TCA. The acidified samples were allowed to cool on ice for at least 30 min, before being centrifuged at 15,000 ×g for 15 min using a Benchtop Centrifuge 5417 R (Eppendorf, Germany). The pellet was resuspended with 6 M NaOH to 50% of the original volume, before being subjected to Nitrogen measurement with a DUMATHERM® Nitrogen analyzer (Gerhardt Analytical Systems, Germany). The value obtained was recorded as TCA-precipitated Nitrogen, and the non-protein Nitrogen (NPN) was then calculated from the difference between the total Nitrogen and TCA-precipitated Nitrogen using the following formula:

\[\text{NPN} (%) = \text{Total Nitrogen} (%) - \text{TCA – precipitated Nitrogen} (%)\]

TCA-precipitated protein content of the RJ, CJ, UJ, and HJC was calculated using a previously described conversion factor of 6.25 (Edwards et al., 1975), using the formula described below:

\[\text{TCA – precipitated protein} (%) = \text{TCA – precipitated Nitrogen} (%) \times 6.25\]

2.3.2. Conductivity and pH

The conductivity and pH of the RJ, CJ, UJ, and HJC samples were measured using sensION™ + EC71 (Hach®, USA) and PHM 220 pH Meter (Radiometer, Denmark), respectively. The sensION™ + EC71 was calibrated biweekly using KCl-based conductivity standard solutions (Hach®, USA). The pH meter was calibrated on a daily basis using IUPAC Certified Buffer Standard Solutions (Radiometer, Denmark).
2.3.3. Size exclusion chromatography (SEC)

The soluble fractions of the centrifugal supernatants from CJ, CJ, UJ and HCJ were freshly analyzed by size exclusion chromatography (SEC) using a Superose 6 Increase 10/300 GL column (Cytiva Life Sciences, Massachusetts, USA) installed on an Akta Purifier Fast Protein Liquid Chromatography System (GE Healthcare, Sweden). A mobile phase of isocratic phosphate-buffered saline (10 mM phosphate buffer, 0.14 M NaCl, pH 7.4) was employed per recommendations from the column manufacturer. The centrifugal supernatants were diluted 2.5× using the mobile phase and passed through a 1.2 μm Phenex™-Glass Fiber 28 mm syringe filter before their manual injection with a syringe with a volume of 100 μL/run. The elution profile was monitored by measuring absorbance at a wavelength of 214 nm. RuBisCO standard from spinach at a concentration of 5 mg/mL was used as a reference for the RuBisCO peak. The untreated control (RJ) was excluded from SEC analyses because of the difficulty to filter the material prior to injection.

2.3.3.1. Peak fractionation and concentration. Based on the elution profile of the RuBisCO standard, the peak attributed to RuBisCO was collected and subjected to further analysis, namely dynamic light scat- tering and Blue Native-PAGE (BN-PAGE). The fractions were diafiltered and concentrated ~5× (based on their initial volume) using Amicon Ultra-0.5 (nominal molecular weight cut-off: 30 kDa) centrifugal filter units (Millipore, Massachusetts, USA) for DLS and SDS-PAGE analyses. For BN-PAGE analysis, the concentration process was repeated as described above (Amicon Ultra-0.5), reaching a ~25× volumetric concentration factor.

2.3.4. Colloidal CBB-stained SDS-PAGE

The SDS-PAGE and CBB-staining procedures were carried out based on Laemmli (1970) and Kang, Gho, Suh, and Kang (2002), respectively. This assay was carried out without standardization of the protein content of the different samples. The original RJ, as well as the supernatants CJ, UJ and HCJ and aliquots from the MF fractions were diluted 5× using MilliQ-water and subsequently mixed with one volume of SDS-PAGE sample buffer (pH 6.8). In the case of the SEC fractions, the samples were mixed directly, with no dilution, with the sample buffer at a ratio of 1:1. The RuBisCO standard was dissolved with MilliQ water: sample buffer (1:1) at a concentration of 1 mg/mL. DTE was added to the sample buffer at a concentration of 20 mM for analysis under reducing conditions. The samples were heated using a Thermomixer C (Eppendorf, Germany) at 90 °C for approximately 2 min, and a volume of 20 μL was subsequently loaded into a 120 C Criterion TGX precast gel. Two lanes of 6 μL PageRuler™ Plus Prestained Protein Ladder were also loaded into the gel. The electrophoresis was carried out using a Power Pac 200 Electrophoresis Power Supply (Bio-Rad Laboratories, California, USA) at a constant voltage of 200 V for approximately 45 min. The gels were then fixed for at least 3 h with a solution of 50% ethanol and 8% phosphoric acid, and stained with colloidal CBB. Imaging was performed using a ChemiDoc™ MP Imaging System (Bio-Rad Laboratories, California, USA).

2.3.5. Protein identification by Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF)

Protein identification was performed as previously described (Jensen, Larsen, & Ropstorff, 1998). In brief, bands of interest were carefully excised from SDS-PAGE gels, and subjected to destaining with 50% acetonitrile and in-gel digestion using Sequencing Grade Modified Trypsin (Promega). The digests were subsequently desalted and concentrated using Paros™ 50 R2 gel-loading tip columns. Thereafter, the samples were eluted onto a MALDI target plate using α-cyano-4-hydroxy-cinnamic acid as matrix. Peptide mass fingerprinting was then performed using an Autoflex Speed (Bruker, Massachusetts, USA). The most abundant ions were then subjected to further fragmentation with tandem mass spectrometry (MS/MS). Database search for both peptide mass fingerprinting and MS/MS were carried out using an in-house Mascot server previously described by Wedholm et al. (2008).

2.3.6. Blue Native-PAGE

A BN-PAGE analysis was performed on the fractions collected by SEC (Section 2.3.3.1), RuBisCO standard, as well as the original RJ, CJ, UJ, and HCJ samples, without standardizing the protein content. The procedure was slightly adapted from the user guide of the NativePAGE™ Sample Prep Kit (Invitrogen™). While the centrifugal supernatants and untreated alfalfa juice were diluted 3× in MilliQ water prior to analysis, the 25× concentrated SEC fractions were not diluted. Aliquots (13 μL) were then added with 5 μL of 4× NativePAGE™ sample buffer (pH 7.2) and 1 μL of MilliQ-water. The RuBisCO standard were dissolved at a concentration of 3.4 mg/mL in a solution of 4× NativePAGE™ sample buffer:MilliQ-water (5:14), and 19 μL of this solution was aliquoted for analysis. The samples were added with 1 μL of NativePAGE™ 5% G-250 sample additive shortly before being loaded into a NativePAGE™ 4–16% Bis-Tris Mini Protein Gel. A lane of 6 μL NativeMark™ unstained protein standards was also loaded into the gel. Pre-chilled cathode and anode buffers were prepared as described in the kit protocol (Invitrogen™). The gel was then run in XCell™ SureLock™ Mini-Cell Electrophoresis system (Invitrogen™, California, USA) at a constant voltage of 150 V for 113 min supplied through a Power Pac 300 Electrophoresis Power Supply (Bio-Rad Laboratories, California, USA). To prevent protein denaturation due to the heat generated by the electrophoresis, the tank was placed in an ice bucket. The gel was stained using Colloidal CBB, as described in the previous section.

2.3.7. Molecular mass determination

The molecular mass (Mw) of proteins present in the CJ, UJ, and HCJ was measured by size exclusion chromatography using a Yarar SEC-4000 column combined with a GFC-4000 (4 × 3.0 mm internal diameter) SecurityGuard Cartridge (Phenomenex®, California, USA) installed on an 1260 Infinity II high-pressure liquid chromatography (HPLC) system (Agilent, California, USA) to a 18-angle DAWN® multi-angle light scattering (MALS) detector (Wyatt Technol- ogy, California, USA). The setup also included a Shodex RI-501 refrac- tive index (RI) detector (Showa Denko K. K., Japan) and an 1260 Infinity II diode array detector (Agilent, California, USA). Samples were eluted with a 50 mM phosphate buffer with 150 mM NaCl and 0.02% sodium azide (pH 7.2) as recommended by Wyatt, at a slow flow rate of 0.5 mL/min and 25 °C. Prior to 30 μL injection by an autosampler at room temperature, samples were diluted 2.5× with the mobile phase and filtered with a 1.2 μm Phenex™-Glass Fiber 28 mm syringe filter. The elution profile was monitored based on the difference in the refractive index between the samples and the elution buffer, and the specific refractive index increment (dn/dc) was set to the 0.1850 mL/g. BSA was used for the normalization and alignment of the detector. Polydispersity of the peaks was estimated by the ratio between the weight average and the number average molecular weight (Mw/Mn). The Mw was calculated using Zimm model with 2nd degree polynomial. Data acquisition and analysis were performed using the ASTRA software version 8.0.0.28 (Wyatt Technology, California, USA).

2.3.8. Particle size analysis

2.3.8.1. Dynamic light scattering. The values of apparent diameter of the particles present in fractions collected from SEC (Section 2.3.3), as well as UJ and HCJ samples were determined by DLS, using a Zetasizer (Malvern Panalytical, UK). Two different dilutions with 10 mM NaCl were prepared to ensure that the results obtained were not affected by concentration: 50× and 100× dilution for heated juice samples, 100× and 200× dilution for ultracentrifuged juice samples, and no dilution and 5× volume reduction for the SEC fractions. The measurements were performed with a PCS8501 cuvette (Malvern Panalytical, UK) with the
exception of the 5 × concentrated SEC fractions, which were measured with a ZEN2112 microcuvette (Malvern Panalytical, UK). Viscosity of the dispersant was set at 0.8872 (mPa·s), and the measurement was performed at a 90° angle. Samples were kept at 25 °C with 10 min of equilibration time.

2.3.8.2. Static light scattering. Particle size distribution of RJ and CJ samples was determined using a Mastersizer 2000 (Malvern Instruments, UK) with diffraction index of 1.45 and 1.33 for particles and water, respectively. Measurements were performed in three technical repeats and were reported as their volume-weighted particle size distribution against mean diameter (d 4, 3). Data acquisition was carried out using the Mastersizer Software version 5.61 (Malvern Instruments, UK).

2.3.9. Moisture analysis

Moisture content of the RJ, CJ, UJ, HCJ, and microfiltered fractions was analyzed using a TGA2 thermal analysis system (Mettler Toledo, Switzerland) based on Wang, Zhang, Fan, Yang, and Chen (2019) with modifications. In brief, approx. 20 µl of samples were weighed into 100 µl aluminum crucibles (Mettler Toledo, Switzerland) and sealed with the appropriate aluminum piercing lids (Mettler Toledo, Switzerland). The samples were heated in Nitrogen atmosphere, at a constant heating rate of 10 °C/min from 35 to 200 °C. Data acquisition was performed using STARes software version 16.30a (Mettler Toledo, Switzerland). The first weight loss from 35 to 140 °C were determined as loss of moisture and volatiles.

2.3.10. Statistical analyses

Analyses of the two separate batches were performed at least in duplicate, and the results were presented as means of results ± standard deviation. An exception to this is the samples obtained from the MF of the CJ (Section 2.2.2), where the experiment was only performed once, the standard deviation presented was therefore from technical repetition. The statistical analyses were carried out using one way analysis of variance, followed with a Duncan’s Multiple Range Test. Statistical computing software R (version 4.0.4) (R Core Team, 2021) was used to perform the statistical analyses with 95% significance level (p < 0.05). The agricolae (version 1.3.3) package was used to perform the Duncan’s Multiple Range Test (De Mendiburu, 2020).

3. Results and discussion

3.1. Pre-treatment

The TCA-precipitated protein, ionic strength, and apparent hydrodynamic diameter of the RJ, CJ, UJ, and HCJ samples are summarized on Table 1. The particle size distribution of the RJ and CJ was analyzed with static light scattering (Mastersizer), as these suspensions contained large particles (>10 µm) and the D4,3 is reported. On the other hand, the apparent diameter for the particles in the UJ and HCJ, was measured by DLS (Table 1). Particle size distribution obtained from the Mastersizer and Zetasizer are presented in supplementary materials S1 and S2, respectively. UJ and HCJ had an average apparent diameter ranging between ~240 and 300 nm. The significant decrease of particle size attributed to the ultracentrifugation and heat clarification treatment might be related to the removal of larger particles present in the green chloroplastic materials, such as cell debris, fibers, and broken chloroplasts (Lamsal, Koegel, & Boettcher, 2003). Table 1 also shows that the average ionic strength (measured by conductivity) was similar between treatments, indicating that changes of ionic composition during the treatments were negligible. The pH values slightly decreased from the raw juice to the three pre-treatments, which were identical to one another. The TCA-precipitated protein was higher for RJ and CJ compared to the UJ and HCJ, due to the removal of the large aggregates by ultracentrifugation and heating. It is important to note that the measurement might be overestimated by inclusion of non-protein Nitrogen compounds that were insoluble by TCA precipitation, for example chlorophyll, which structurally contains 4 nitrogen atoms, was visibly co-precipitated with proteins during TCA precipitation of RJ and CJ samples.

The SDS-PAGE protein profiles of the RJ, CJ, UJ, and HCJ was mainly comprised of bands at ~55 and ~15 kDa (Fig. 2). These bands were confirmed to be the large and small subunits of Rubisco using peptide mass fingerprinting and MS/MS using MALDI-TOF (Supplementary Material S3). The differing pre-treatments were shown to slightly affect the migration of the large Rubisco subunit, suggesting a slight shift to a smaller size in the UJ and HCJ samples. Furthermore, a small band at ~10 kDa was observed in RJ and CJ, which was not as prominent in UJ and HCJ samples. This indicates that this small molecular weight polypeptide band was largely removed by both ultracentrifugation and heat treatments. It is worth noting that the band was green colored prior to fixing and staining, indicating its association with chloroplastic materials.

3.2. Microfiltration

The visual appearance of the juice after different treatments is shown in Fig. 3. The original alfalfa juice was green, as well as its corresponding CJ, clarified by low speed centrifugation. On the other hand, most of the green color was removed through ultracentrifugation, as well as with the mild heating treatment at 50 °C for 30 min, followed by low speed centrifugation.

When the treatments were subjected to the MF process, the CJ retentate retained the green color, while the permeate was clear. UJ and HCJ samples were clearer in appearance with HCJ samples appearing slightly greener. The clearer appearances of the UJ and HCJ samples compared to the CJ were also observed in their corresponding permeates and retentates, due to the precipitation of the green material during centrifugation. The UJ retentate showed a slightly yellow color, while the HCJ retentate showed a green hue, suggesting the retention and concentration of green pigments in the fraction.

To evaluate possible differences in protein permeation through the MF membrane, the retentate and permeate fractions were analyzed using SDS-PAGE (Fig. 4). Minimum differences under reducing (Fig. 4) and non-reducing (data not shown) conditions were observed for all samples, showing the same intensity of bands of the large (~55 kDa) and small (~15 kDa) subunit of Rubisco. It was clearly observed that the concentration of the proteins decreased overtime in the CJ and UJ permeates (Fig. 4A and B). On the other hand, the protein in the permeate for the HCJ sample remained constant throughout the filtration process, indicating free permeation through the membrane (Fig. 4C).

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<thead>
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<th>Table 1</th>
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<td>Apparent diameter, ionic strength, and TCA-precipitated protein content of untreated alfalfa juice and centrifugal supernatants from three different treatments.</td>
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<tr>
<td>Raw juice</td>
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<tr>
<td>Average apparent diameter (µm)</td>
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<tr>
<td>Ionic strength (mS/cm)</td>
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<tr>
<td>pH</td>
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<td>TCA-precipitated protein (%)</td>
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a Average apparent diameter for raw and clarified juices were measured by static light scattering (Mastersizer 2000), while results from ultracentrifuged and heat clarified juice were measured by dynamic light scattering (Zetasizer).

b pH of raw juice was adjusted as part of the processing (Section 2.2.1).
As shown in Fig. 4A, removing insoluble material through centrifugation (CJ) was not sufficient to have an efficient separation, and a low amount of protein was transmitted through the membrane with a nominal cut-off size of 1 μm (Fig. 4). This was most probably due to the large particles still present in the CJ sample (Table 1), which irreversibly fouled the filters irrespective of the vibration that was applied in the cross flow system. The decrease of the RuBisCO permeation over time in CJ samples was in agreement with Eakin et al. (1978), who observed a ~50% decrease in protein permeation through a 0.2 μm membrane after a short time of filtering alfalfa juice that was pre-filtered with nylon bag to remove insoluble fibers. Furthermore, the authors observed the formation of a significant gel layer, which compromised the selectivity of the membranes. Fouling was also observed by Zhang et al. (2015), during microfiltration of alfalfa juice using different filters.

Fig. 4 shows that there was a higher extent of protein permeation for UJ (B) and HCJ (C) than CJ (A). These results correspond well with the apparent hydrodynamic radius of the particles (Table 1) in the UJ and HCJ, which were ~25–30% of the nominal cut-off value (Table 1) of the filtration membrane. However, there was a difference between UJ and HCJ, as at the beginning of the filtration process of UJ the protein permeation appeared to be higher relative to the other timepoints of the filtration. This result implied that the supramolecular structure of the protein in the two samples may be different, and that the extent of the molecular interactions during the MF of UJ was higher than for the protein in HCJ, which continued to permeate. Since the green chloroplastic material was mostly removed from both UJ and HCJ, these interactions might be attributed to a large extent to RuBisCO and the residual soluble components. The MF results of the HCJ are in agreement with that of Nieuwland et al. (2021), who successfully employed mild heat treatment as pre-treatment for a tandem MF-UF process on duckweed protein. It is also worth noting that other proteins in the juice (i.e. ~40 and 20 kDa) also partitioned differently due to the different pre-treatments. These proteins appeared to permeate through the membranes in the UJ and HCJ treatments, but were absent in the permeate of CJ. The permeation of the protein seemed to have a similar trend to the flux of the process (supplementary material S4), as the HCJ was shown to be highest in all differing timepoints, followed by the UJ, with the CJ being the slowest. Some degree of flux decline over time was observed in all treatments, implying that fouling and gel layer formation occurred to some extent during the process. The flux during processing of HCJ was reduced by ~3 fold when vibration was not performed (data not shown), indicating that the fouling during processing was minimized by the presence of vibration. Despite the slower flux over time during processing, a water flux of ~400 L/m²/h was recoverable in all treatments, except for CJ, where ~75% of the water flux was lost even after CIP.

The dry matter concentration of the various fractions obtained by microfiltration is summarized in Table 2. While the dry matter of the UJ and HCJ samples did not change during the MF process, showing the similar values for the feed, retentate, and permeate samples, the dry matter of the CJ was shown to be concentrated on the retentate side. Table 2 also shows the amount of TCA-precipitated protein and NPN present in the MF fractions. In CJ, the TCA-precipitated were shown to be lower in the permeate side despite having similar values in the feed and retentate samples, indicating a possible loss of protein, potentially due to deposition on membrane surfaces. Furthermore, the TCA-precipitated protein of the UJ was shown to be concentrated on the retentate side, which is in agreement with Fig. 4B. TCA-precipitated protein values of the feed, retentate, and permeate of HCJ appeared to be a plateau, indicating that the proteins were able to pass through the MF membranes upon heat treatment, which is in good agreement with Fig. 4C. It is reiterated that the protein concentration obtained through TCA-precipitation are rough estimates due to the use of a conversion factor of 6.25 (Maehre, Dalheim, Edvinsen, Ellevoll, & Jensen, 2018), as well as the aforementioned potential overestimation of crude protein in CJ samples due to co-precipitation of chlorophyll during TCA-precipitation.

While the NPN value of the CJ permeate was higher than those of the feed and retentate, the NPN values of the UJ and HCJ were shown to be relatively on a plateau during the MF process. This indicates that the NPN can freely pass through the membranes both in the absence of (in the case of UJ and HCJ) and despite the presence of fouling (for CJ), therefore emphasizing that total nitrogen value would not be a good indicator for protein distribution for the alfalfa juice. It is worth mentioning that the NPN values (Table 2) reported are considerably high. However, the amount of non-protein nitrogen in alfalfa has been shown to be high (~28%) early on (Miller, 1921), and a recent study has demonstrated that twin screw pressing extracted higher portion of NPN, than protein/amino acid-associated Nitrogen (Damborg, Stokkilde, Jensen, & Weisbjerg, 2018). Ultimately, it should be noted that the NPN calculation method does not distinguish between nitrogen-containing compounds that are associated with proteins, e.g. free peptides and amino acids, and those that are not, e.g. urea and ammonia.
Fig. 3. Visual appearance of the juice samples subjected to clarification (CJ), ultracentrifugation (UJ), heating (HCJ), and subsequently microfiltration processes using 1 μm hydrophilized PTFE membrane in a vibrating, cross-flow Vibro-Lab35P system.

Fig. 4. SDS-PAGE under reducing conditions for clarified (A), ultracentrifuged (B), and heat clarified (C) juice samples, and their microfiltered fractions. Start: permeate at beginning of filtration; Middle: permeate at approximately 1.5× volumetric concentration factors; End: permeate at 2× volumetric concentration factors.

Table 2
Dry matter, TCA-precipitated protein, and non-protein Nitrogen of filtration feed, retentate, and permeate of clarified, ultracentrifuged, and heat clarified juice samples. Within the same pre-treatments, superscripts denote significant difference at \( p < 0.05 \) among different fractions.
3.3. Molecular details of juice samples

The SEC and BN-PAGE measurements were carried out on freshly produced samples in order to avoid unwanted aggregation due to freezing. The SEC elution profile (Fig. 5A) showed a small excluded volume peak for CJ and UJ at ~9 mL. The CJ, UJ, and HCJ samples also exhibited a RuBisCO peak eluting at ~15 mL, matching the major peak of the RuBisCO standard. The peaks eluting after 18 mL are likely to be phenolic compounds due to their smaller hydrodynamic radius, high absorbance at 320 nm (wavelength related to phenolic compounds) (Kaeswurm, Scharinger, Teipel, & Buchweitz, 2021), a yellowish color after fractionation, and low light scattering signal in the MALS detector (data not shown). Furthermore, we have previously tried to identify such peak with SDS-PAGE without succeeding in obtaining bands (Tanambell et al., 2022).

The identity of the peak eluting at ~15 mL was further confirmed by their ~500 kDa size in the BN-PAGE (Fig. 5B). The band at ~500 kDa is roughly in agreement with the RuBisCO band from alfalfa observed by Ladig et al. (2011), as well as the known size of native size of RuBisCO from higher plants (Di Stefano et al., 2018). It is interesting that the BN-PAGE showed no well-defined RuBisCO band in the unfractionated RJ and CJ samples despite the fact that an intense RuBisCO peak was observed on the SEC (Fig. 5A) and SDS-PAGE (Fig. 2) profiles of the CJ. In addition, the SEC fraction collected from CJ showed a strong RuBisCO band in the BN-PAGE (Fig. 5B), suggesting that dilution with the salt-phosphate buffer and the fractionation through the column might have eliminated some of the contaminants and/or aggregates affecting the electrophoresis. The wells of the unfractionated RJ, CJ, UJ, and HCJ samples also depicted some larger aggregates that did not migrate into the gels (solid box in Fig. 5B). HCJ samples showed the least intensity of such band, and this might correspond to its SEC profile (in Fig. 5A), which lacked a peak at ~9 mL present in CJ and UJ samples. This indicates that the mild heating process might have either dissociated or precipitated these aggregates.

As the fractions were demonstrated to be comprised of native RuBisCO (Fig. 5B), the SEC fractions were subjected to hydrodynamic size analysis by DLS. The average particle size of the SEC-fractionated RuBisCO peaks of the CJ, UJ, and HCJ had similar values of 15.03 ± 0.92, 15.12 ± 0.76, and 15.53 ± 0.49 nm, respectively. These values are similar to that shown by Desai et al. (2014). When comparing the particle size of these purified RuBisCO fractions to the values reported in Table 1, it is clear that the juice samples are comprised of larger mixed particles, which might affect the filtration of the protein through the membrane.

The CJ, UJ, and HCJ samples were also analyzed using SEC-MALS-RI. The normalized RI signal and molar mass are plotted against elution volume in Fig. 6. The peak eluting at 5.5 mL was observed in CJ and UJ, but not HCJ samples, as already seen in Fig. 5A. The peak had a polydispersity index of ~1.2 in both samples, indicating that the peak is likely comprised of aggregates. The molecular mass (Mw) of the peak was 170 ± 17 and 176 ± 33 MDa for CJ and UJ, respectively (not shown in graph to emphasize clarity to the Mw of the RuBisCO peak). The peak eluting at ~8.25 mL, containing RuBisCO, was monodisperse (polydispersity index of ~1.0), and showed a very different average Mw value was obtained from the RuBisCO peak of the CJ (703.3 ± 48.9 kDa) when compared to those of the UJ and HCJ samples (533.5 ± 28.3 and 517.3 ± 23.2 kDa, respectively). The similarity of the peaks on the SEC retention time indicates that the Mw shift did not provide significant influence to the hydrodynamic volume of the RuBisCO protein.

The Mw obtained from the RuBisCO peak of UJ and HCJ corresponded to the bands observed in the BN-PAGE (Fig. 5B), and also in agreement with the ~497 kDa value reported by Tomimatsu (1980). However, the larger (~700 kDa) value obtained for RuBisCO peak from CJ (Fig. 6) has never been reported before, and indicates a non-covalent association of RuBisCO with other components in the CJ. These results are in agreement also with the SDS-PAGE profile (Fig. 2), which showed that the ultracentrifugation and heat treatments shifted the large subunit towards a smaller size. However, this difference in the size of the RuBisCO was not observed in the BN-PAGE, when the protein was isolated by size exclusion (Fig. 5B; lane number 4 from the right), possibly due to the concentration and diafiltration used during the sample preparation for BN-PAGE (see Section 2.3.3.1), which might have removed some compounds associated with CJ that were measured in the MALs.

Ladig et al. (2011) have visualized complexes of ~480 and ~750 kDa from pea chloroplasts by two different conditions of native-PAGE, and confirmed that the bands were comprised of RuBisCO through mass spectrometry. Nonetheless, only a RuBisCO complex of ~480 kDa was found from alfalfa chloroplasts in the same study. These differences might be attributed to the fact that unlike apparent Mw obtained from migration behavior in PAGE gel systems, the Mw measurement by SEC-MALS does not require detergent and is less affected by the presence of other charged compounds. There were also differences in the alfalfa parts that we used in the present study compared to Ladig et al. (2011), who isolated chloroplasts from alfalfa leaves, whereas stems were pressed together with leaves in this study (Section 2.2.1). To the best of our knowledge to-date, there are no published data on the absolute

![Fig. 5. (A) SEC chromatograms of RuBisCO standard, clarified (CJ), ultracentrifuged (UJ), and heat clarified (HCJ) juice samples and (B) BN-PAGE of RuBisCO standard, CJ, UJ, HCJ, their SEC fractions, and RJ samples. Curves in fig. A are average of two separate batches; solid box in fig. A exemplifies a peak that was present only in CJ and UJ samples; dashed box in fig. A indicates the RuBisCO peaks that were fractionated for further analysis; solid box in fig. B exemplifies that some proteins did not migrate into the gels.](image-url)
molecular mass of RuBisCO from alfalfa as measured by light scattering except of that reported by Tomimatsu (1980), and for the first time, the difference in the \( M_w \) of the protein depending on the treatment of the juice has been reported. Ultimately, further study will be required to characterize these larger RuBisCO complexes and their constituents.

### 3.4. General discussion

The present work clearly demonstrated that despite of sodium sulfite addition to inhibit oxidation of polyphenols and their subsequent reaction with proteins, RuBisCO in the alfalfa juice exists as non-covalent aggregates, rather than as singular hexadecamers. The possibility of using MF for partitioning the green color in the retentate is an advantage for using the permeate for downstream food applications where green color is not desired, such as egg white analogs (Zhou, Vu, & McClements, 2022). Further processing is, however, required for making the permeate adequate for food, not solely for concentrating the protein, but also to remove excess sulfite and anti-nutrients in the system. Various processes that can be considered for such purpose include UF-dialfiltration (Knuckles, Edwards, Miller, & Kohler, 1980), ion exchange expanded bed chromatography, or thermal denaturation (Stødkilde, Ambye-Jensen, & Jensen, 2021).

The MF results demonstrated that mild heating was a superior pre-treatment relative to clarification and ultracentrifugation for MF, allowing RuBisCO from alfalfa juice to pass freely through membranes with a nominal cut-off value of 1 \( \mu \)m more freely up to 2 \( \times \) volumetric concentration factor. However, it needs to be acknowledged that the mild heating process also potentially caused more protein loss (Table 1). This is in agreement with a previous study which also reported that alfalfa HCJ produced with a similar manner only had ~50% the nitrogen of the green juice (Nynás et al., 2021). This possible protein loss is not necessary wasted, as the green extraction paste produced from the heat treatment can be potentially utilized for animal feed (Møller et al., 2021).

The methodology used for the mild heating process, including thermal processing, low speed centrifugation, and MF, are also relatively more scalable than other frequently used purifications means, such as ultracentrifugation or column chromatography (Di Stefano et al., 2018). However, proteolysis needs to be considered when scaling up the process as Koschuh et al. (2004) has demonstrated that protein degradation in alfalfa juice is rapid at room temperature. Furthermore, an endoprotease from alfalfa has been demonstrated to be present in solution and active after 1 h of ultracentrifugation at 100,000 \( \times g \) (Ferrari, Alpi, & Balestreri, 1988). Considering these past endogenous proteolytic activity reports on alfalfa extracts, further research on approaches for minimizing protein degradation during processing is needed for scaling up the whole process. Another issue to be considered in scaling up is the regular process control for protein quantification, in which we have demonstrated that total Nitrogen does not represent the protein distribution of different fractions of alfalfa juice. Ultimately, the economical feasibility of the whole system does not only rely on protein as single output from processing alfalfa, but will also depend on the valorization of side streams, such as pulp after screw pressing and leftover liquid fractions generated from processing, for other purposes as suggested in Møller et al. (2021).

### 4. Conclusion

This work assessed the partitioning behavior of RuBisCO from alfalfa following three different pre-treatments. It was demonstrated that ultracentrifugation and mild heat treatment were able to separate the soluble RuBisCO proteins from the chloroplastic materials, while low speed centrifugation was unsuccessful. RuBisCO from CJ samples was shown to possess higher \( M_w \) than those of the other samples, which were similar in values with the known \( M_w \) of RuBisCO of higher plants. In addition, the CJ and UJ samples were shown to contain nanosized aggregates not present in HCJ. Interestingly, only the mild heat treatment was able to allow the soluble RuBisCO to pass through membranes with 1 \( \mu \)m cut-off value. Although further studies in optimizing the mild heat and MF conditions to minimize the protein loss and maximize removal of chloroplastic materials are needed, this study demonstrated that mild heating can be used to manipulate the supramolecular structure of RuBisCO from alfalfa, while preserving its native state, allowing its fractionation using MF.

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**Fig. 6.** Size exclusion chromatograms exhibiting normalized refractive index (RI) and molar mass versus elution time of clarified, ultracentrifuged, and heat clarified juice samples.

\( \text{M}_w: \) molecular weight; \( \text{RI}: \) refractive index; curves are average of duplicates; black box indicates aggregate peaks that were present in CJ and UJ samples.
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CRediT authorship contribution statement

Hartono Tanambell: Conceptualization, Methodology, Investigation, Writing – original draft. Anders Hauer Møller: Conceptualization, Methodology, Supervision, Writing – review & editing. Laura Roman: Methodology, Writing – review & editing. Milena Corredig: Conceptualization, Methodology, Supervision, Writing – review & editing. Trine Kastrup Dalsgård: Conceptualization, Methodology, Supervision, Writing – review & editing. Funding acquisition.

Declaration of competing interest

The authors declare no conflict of interests.

Data availability

Data will be made available on request.

References


