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Recovery of protein from green leaves: Overview of crucial steps for utilisation

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ABSTRACT

Plant leaves are a major potential source of novel food proteins. Till now, leaf protein extraction methods mainly focus on the extraction of soluble proteins, like rubisco protein, leaving more than half of all protein unextracted. Here, we report on the total protein extraction from sugar beet leaves (*Beta vulgaris* L.) by a traditional thermal extraction method consisting of mechanical pressing, heating to 50 °C and centrifugation. The resulting streams (i.e. supernatant, green-protein pellet and fibrous pulp) were characterised in terms of composition, physical structure and processing options. The protein distributed almost equally over the supernatant, pellet and pulp. This shows that thermal precipitation is an unselective process with respect to fractionation between soluble (rubisco) and insoluble (other) proteins. About 6% of the total protein could be extracted as pure rubisco (90% purity) from the supernatant. Surfactants commonly used for protein solubilisation could hardly re-dissolve the precipitated proteins in the pellet phase, which suggested that irreversible association was induced between the co-precipitated proteins and cell debris. Thus, the extraction of this protein will require prevention of their co-precipitation, and should take place in the original juice solution.

1. Introduction

Leaf proteins could potentially form a major protein source for food application. Leaves from several crops have been considered depending on their protein content, regional availability, social needs and current uses (e.g. alfalfa, spinach, grass, cassava, moringa, tobacco). Other sources include leaves that are available as by-products from certain crops on large scale (e.g. cassava, barley, broccoli, sugar beet). Despite many decades of leaf protein study and the broad portfolio of suitable crops, the existing extraction processes have not reached industrial production for human food application yet. An important reason could be the focus on soluble proteins, thereby neglecting the great potential of the other proteins present in leaves. It is therefore important to further explore how the remaining proteins can be utilised as well, which is the purpose of this study.

Traditional leaf protein extraction methods focus on soluble protein recovery in the form of leaf protein concentrate (LPC). Soluble LPC accounts for about half of the leaf proteins and it is the preferred protein fraction for human food applications due to its white colour and lack of off-flavour (Barbeau & Kinsella, 1988). The enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco) accounts for most of the solubilised protein and it can be purified from the soluble leaf protein fraction. During LPC production, the final protein yield is typically 40–60% of the total leaf protein and it depends on the plant source and extraction process (Bals et al., 2012; Chiesa & Gnansounou, 2011; Dijkstra, Linnemann, & van Boekel, 2003).

Leaf protein extraction involves three major steps: tissue disruption by mechanical treatments, protein precipitation by one or more heat and/or pH treatments and protein concentration (Coldebella et al., 2013; Edwards et al., 1975; Jwanny, Montanari, & Fantozzi, 1993; Merodio & Sabater, 1988). For instance, rubisco purification from soluble leaf concentrate often combines different methods such as heat precipitation at 80–82 °C, pH precipitation, flocculation and organic solvent precipitation (Barbeau & Kinsella, 1983, 1988; Martin, Nieuwland, & De Jong, 2014; Van de Velde, Alting, & Pouvreau, 2011). The combination of steps allows protein fractionation to some extent as well as removal of the undesired green colour.

LPC and rubisco by-products contain the remaining leaf proteins. These by products are the leaf pulp (rich in fibres) and a

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green insoluble curd. In general, rubisco isolation methods are designed to precipitate undesired components to the green curd, which contains cell debris, broken chloroplast, pigments and particulates (Lamsal, Koegel, & Boettcher, 2003). The proteins in the curd are often referred as green-protein fraction. So far, main focus was on the nutritional value of this protein fraction for animal feed applications rather than exploring further processing options and protein recovery for high value applications such as food (Byers, 1971; Chiesa & Gnansounou, 2011; Merodio, Martín, & Sabater, 1983).

The fact that rubisco and LPC side streams are still rich in proteins explains the low protein yield of the extraction processes. Leaf protein production for food is not yet economically feasible at large scale, though coupling to biofuel production in a larger biorefinery operation might positively influence the economic feasibility (Bals & Dale, 2011; Bals et al., 2012; Chiesa & Gnansounou, 2011). The use of rubisco side streams for high value applications such as food proteins has not been extensively reported. A study with tobacco leaves described the extraction of green-proteins using harsh conditions such as alkaline pH, organic solvents and high temperatures, recovering half of the protein from the tobacco biomass (Teng & Wang, 2011). A similar approach for protein recovery would not only improve the feasibility of rubisco production, but would also enhance the use of existing bio-resources; although milder extraction conditions are necessary to preserve protein functionality and achieve a food grade application.

The aim of this study is to consider options for protein extraction from side streams of the rubisco production and to analyse the effects of the initial extraction steps on the overall protein yield and the downstream processes. Rubisco production processes have been extensively developed and optimised for several crops; therefore, it is a relevant starting point to explore the used of the valuable side streams. Sugar beet leaves were used for this study due to their large availability and the lack of extensive research on protein extraction from those leaves. The resulting fractions after thermal extraction were characterised in terms of composition, physical structure, functional properties and processing options. This information was used to evaluate the feasibility of two processing scenarios: further protein recovery from side streams after rubisco extraction and total protein recovery from leaves. The latter is focused on obtaining functional leaf fractions rather than single-protein isolation and considers the need for re-thinking the leaf biorefinery approach that has been followed for several decades.

2. Materials and methods

2.1. Leaf material and protein extraction

Sugar beet leaves (SBL) were harvested from a sugar beet production field in Wageningen, The Netherlands. The leaves had an average dry matter content of 12% (wt) and the average composition in dry basis was 14.8 wt% protein, 2.9 wt% fat, 30.3 wt% total dietary fibre and 20 wt% ash. The leaves were washed with cool tap water (~10 °C; hardness 4.4 degrees German hardness, dGH) and dried with towel-paper before processing the leaves with a screw press, Angelia juicer II 7500 (Angel Juicers, Queensland, Australia). Pressing gives a layer of fibres around the screws inside the press barrel, acting as filtering bed. A green juice is expressed, while the fibrous pulp is extruded out at the end of the screws.

Leaf proteins were extracted from the juice by heat precipitation (50 °C, 30 min) as described by several authors for rubisco and LPC extraction protocols (Jwanny et al., 1993; Martin et al., 2014; Merodio et al., 1983; Rathore, 2010; Sheen, 1991; Telek, 1983). The heated juice was centrifuged at 15,000*g* for 30 min and carefully separated into supernatant and pellet.

2.1.1. Consecutive washing

The SBL green pellet remaining after centrifugation was washed three times with tap water for additional recovery of the soluble protein that remained in the interstitial fluid. The washing steps were done with a pellet-to-water weight ratio of 1:20 and centrifuged at 7000*g* for 30 min. The resulting supernatant and pellet were analysed for composition and mass distribution.

2.1.2. Solubilisation with surfactants

The pellet obtained after heat precipitation was freeze dried and stored before processing. The dried pellet was re-suspended in different surfactant solutions or water as control. The surfactants tested were Triton X-100 (p-(1,1,3,3-tetramethylbutyl)-phenyl ether, octyl phenol ethoxylate), Zwittergent 3-12 (n-Dodecyl-n,n-dimethyl-3-ammonio-1-propanesulphonate) and CHAPS ((3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulphonate); all from Sigma–Aldrich (St. Louis, MO, USA) at the same concentration (0.3 wt%) and two pellet concentrations were compared 0.3 wt% and 5 wt%. The final mix was centrifuged at ~4800*g* for 20 min. The resulting supernatant and pellets were analysed for composition and mass distribution.

2.2. Compositional analysis

The dry matter content was determined by oven drying at 50 °C for 48 h and the weight losses were recorded. Protein nitrogen was determined by Dumas analysis with a NA 2100 Nitrogen and Protein Analyser (ThermoQuest-CE Instruments, Rodeno, Italy). Methionine was used as standard during the analysis. Duplicate measurements were made for all samples. A conversion factor of 6.25 was used to convert nitrogen values to protein; this factor is commonly used in previous leaf protein studies (Fantozzi & Sensidoni, 1983; Hernández, Martínez, Hernández, & Urbano, 1997; Sheen, 1991; Teixeira, Carvalho, Neves, Silva, & Arantes-Pereira, 2014; Teng & Wang, 2011; Zanin, 1998). The concentration of soluble protein was determined by BCA assay (Smith et al., 1985), using Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, US), in which BSA was used as standard. The lipid content was determined by Soxhlet extraction with petroleum ether, according to AACC method 30-25 (AACC, 1983b). The samples were dried and ground before the extractions. Total dietary fibre was determined according to AACC method 32-05.01 using Megazyme assay kit K-TDFR (Megazyme International, Bray, Ireland). The ash content was determined according to the AACC official method 08-01 (AACC, 1983a).

2.3. Microscopy

Microscopic images of the SBL products were made with a Nikon Microphot FL microscope equipped with a Photometrics CCD camera (Photometrics®, Tucson, USA). Samples were placed directly on a glass slide, covered with a normal cover glass and observed using normal light, polarised light, and fluorescence with a Nikon B-3A fluorescence filter to detect chlorophyll autofluorescence (λ_{exc} = 440 nm; λ_{em} = 685 nm). Rhodamine B was used for protein staining.

2.4. SDS-page

The protein characterisation of the samples was done by reducing SDS polyacrylamide gel electrophoresis, using a Bio-Rad Mini-Protean cell (Bio-Rad Laboratories Inc., Hercules, USA). The samples were diluted with sample buffer (62.5 nM Tris–HCl, pH 6.8; 2 w/v% SDS; 5 w/v% 2-mercaptoethanol). The weight ratio of sample-to-buffer was 1:4 for pellet samples and 1:1 for supernatant and juice samples. Before electrophoresis, each sample was heated to 95 °C for 5 min in a heating block and then centrifuged at 10,000*g* for