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Understanding leaf membrane protein extraction to develop a food-grade process

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ABSTRACT

Leaf membrane proteins are an underutilised protein fraction for food applications. Proteins from leaves can contribute to a more complete use of resources and help to meet the increasing protein demand. Leaf protein extraction and purification is applied by other disciplines, such as proteomics. Therefore, this study analysed proteomic extraction methods for membrane proteins as an inspiration for a food-grade alternative process. Sugar beet leaves were extracted with two proteomic protocols: solvent extraction and Triton X-114 phase partitioning method. Extraction steps contributed to protein purity and/or to selective fractionation, enabling the purification of specific proteins. It was observed that membrane proteins distributed among different solvents, buffers and solutions used due to their physicochemical heterogeneity. This heterogeneity does not allow a total membrane protein extraction by a unique method or even combinations of processing steps, but it enables the creation of different fractions with different physicochemical properties useful for food applications.

1. Introduction

Green leaves are considered as an underutilized protein source for food applications. A potential leaf source is leaves from sugar beet plants (*Beta vulgaris* L.), since the leaves can be regarded as edible (Lim, 2016) and constitute an abundant waste stream. Total protein extraction from sugar beet leaves would deliver 450–600 kg/ha, which is comparable to soy (450–600 kg/ha) and cereals (~570 kg/ha) (van Krimpen, Bikker, van der Meer, van der Peet-Schwering, & Vereijken, 2013). However, the use of leaf proteins, not only from sugar beet plants, is hindered by the lack of extraction processes that can reach high yields (Bals et al., 2012). So far, the development of protein extractions processes from leaves have focused on the soluble protein fraction, leaving the insoluble fraction in side streams (Tamayo Tenorio, Gieteling, de Jong, Boom, & van der Goot, 2016). Therefore, a suitable extraction process for these neglected proteins is needed to increase the current protein yield, to deliver food quality proteins and ultimately to optimise existing resources.

Leaf insoluble proteins mainly consist of membrane proteins, and their lack of solubility in water hinders extraction and subsequent utilisation. These proteins are removed in the extraction processes available for food applications (Lamsal, Koegel, & Gunasekaran, 2007). By discarding the membrane proteins, not only is the intense green colour of leaf proteins removed, but also half of the proteins. To utilise the leaves better, processes for the extraction of the membrane proteins are required. Other disciplines such as proteomics, photosynthesis biology and plant membrane dynamics utilise different solubilisation strategies to analyse these proteins. Thus, the extraction methods developed for analytical purposes can provide insights into how to extract membrane proteins.

Proteomic protocols are optimized to extract all types of membrane proteins, even in their native state because of their important roles in several cell functions. Isolation of membrane proteins means detaching the proteins from the lipid membrane and breaking the interactions (electrostatic, van der Waals, hydrogen bonds) with enzyme cofactors, chlorophylls and other pigments. These interactions and the presence of interfering compounds, make leaf and stem material more challenging for protein extraction compared to tissue from other organisms such as yeast or mammals (Wang, Tai, & Chen, 2008). Even if proteomic protocols are meant to achieve complete extraction, they cannot be directly translated to a preparative, industrial scale process, since they use extraction media that would not be allowed on larger scales. However, the protocols do generate an understanding of the interactions that are at play, providing valuable insights for large-scale extraction using other media.

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The objective of this study is therefore to learn from proteomic protocols on possible extraction routes for leaf membrane proteins, and evaluate food-grade equivalent options through understanding the role of each extraction step/reagent and the behaviour of the membrane proteins during extraction. Two proteomic extraction methods were explored with sugar beet leaves: a protocol designed especially for membrane protein purification, and a phase partitioning protocol using Triton X-114, designed for integral membrane protein isolation. The effect of each extraction step was analysed in terms of total protein distribution and chlorophyll removal. The knowledge gained through these proteomic methods was used to assess the possibilities for membrane protein extraction and to explore food-grade alternatives depending on the final application. Understanding these methods gave insights on the technical feasibility of a food-grade process aimed for large scale implementation.

2. Materials and methods

2.1. Chemicals

Ammonium acetate, phenol, TCA (trichloroacetic acid), SDS (Sodium dodecyl sulphate), Triton X-114 ((1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol), HCl (hydrogen chloride), Tris-HCl, 2-mercaptoethanol and the PBS components (Na_2HPO_4, KH_2PO_4, NaCl, KCl) were purchased from Sigma-Aldrich Co. LLC. (St. Louis, USA). Pre-Stained Protein Standard and 12% Tris–HCl SDS-ready gel were purchased from Bio-Rad (Bio-Rad Laboratories Inc., Hercules, USA) Acetone and methanol were purchased from Actu All Chemicals b.v. (Oss, The Netherlands).

2.2. Plant material

Sugar beet leaves (SBL) were collected from a sugar beet field (0.85 ha) near Wageningen, The Netherlands. The field belongs to Wageningen University. The leaves had an average dry matter content of 10.3 ± 0.4 wt% and a protein content of 19.4 ± 1.9 wt% on dry basis. Before processing, the leaves were washed with cool tap water (~10 °C; hardness 4.4 degrees German hardness, dGH) and dried with paper-towel.

2.3. Proteomic extraction protocols

Two proteomic protocols were considered in this study to observe the protein distribution under different conditions. Proteomic studies usually combine several methods, which complement each other and result in identification of more proteins (Friso et al., 2004; Kamal et al., 2013).

2.3.1. Solvent extraction protocol

A standard proteomic extraction protocol was used as described by Wang, Vignani, Scali, and Cresti (2006), and the sample size was increased up to 10 g to allow mass and protein balance calculations. SBL were pulverised with liquid nitrogen and mixed with 10% TCA/acetone in a powder to liquid ratio of 1:10, followed by centrifugation (step 1). After discarding the supernatant, 80% methanol with 0.1 M ammonium acetate was added to the pellet in a 1:10 ratio, mixed and then centrifuged (step 2). The supernatant was again discarded, replaced with 80% acetone in a pellet to solvent ratio of 1:10, mixed and centrifuged (step 3). The supernatant was discarded and the pellet was air dried. Afterwards, 6 ml/g pellet of 1:1 phenol/SDS buffer was added, mixed and incubated for 5 min at room temperature to allow phase separation (step 4). After centrifugation, the phenol phase was transferred to a new centrifuge tube. The tube was filled with 80% methanol with 0.1 M ammonium acetate in a 10:1 ratio with the phenol phase. The mixture was stored over night at −20 °C and then centrifuged (step 5). The white pellet was collected and washed consecutively with 100% methanol (step 5) and 80% acetone (step 6), followed by centrifugation each time. The final pellet was air dried. All mixing steps where done by vortexing at room temperature and the centrifugation steps were done at 16,000*g* at 4 °C for 3 min. From the mass and protein balance, the protein yield was calculated as grams of protein in the sample per grams of protein in the starting leaf material.

2.3.2. Phase partitioning protocol

The phase partitioning with Triton X-114 was done based on two methods: according to Brusca and Radolf (1994) and Okamoto, Schwab, Scherer, and Lisanti (2001). SBL were also ground in liquid nitrogen. The leaf powder was mixed with a Titron X-114 solution (2% final concentration) in PBS (pH 7.4) to a surfactant to protein ratio of 10:1, using a rotor-stator IKA T25 (IKA-Werke GmbH & Co. KG, Staufen, Germany) at 6,500 rpm for 30 s, and at 9,500 rpm for 30 s. The mixture was centrifuged (13,000*g*, 4 °C, 10 min) to remove the cell debris. The supernatant was incubated at 37 °C for 10 min and centrifuged at 13,000*g* for 10 min. The surfactant (bottom) phase was analysed for composition and protein characteristics. The aqueous (top) phase was transferred to a new tube and mixed again with Titron X-114 solution in a 1:1 weight ratio, incubated and centrifuged under the same conditions. Samples were taken at each extraction step to establish mass and protein balances and to calculate the protein yield as previously indicated. The resulting phases were analysed for their composition and protein characteristics (SDS-page). Before compositional analysis, the fractions were dried in a convective oven at 105 °C for 24 h.

2.4. Food-grade extractions

Fresh SBL were pressed through a twin screw press Angelia juicer II 7500 (Angel Juicers, Queensland, Australia). The juice was used for protein extraction, while the fibrous pulp was extruded out at the press and discarded.

2.4.1. pH precipitation

The initial juice's pH was 6.1 and it was lowered to pH 3.5 and 4.5 with a 1 M HCl solution. The juice was incubated under continuous stirring for 1 h and centrifuged at 7,000*g* for 10 min at room temperature. Both the pellet and supernatant were collected for compositional analysis. Samples were done in triplicate.

2.4.2. Successive acetone washes

SBL juice was heated to 50 °C for 30 min and centrifuged at 15,000*g* for 30 min. The resulting green pellet was freeze-dried and stored until used. The dry pellet was washed with 80% acetone for 5 times, using a powder to solvent ratio of 1:20. At each washing step, the mixture was incubated at −20 °C for 1 h, followed by centrifugation at 4,816*g* for 20 min, at 4 °C. The supernatants were analysed for chlorophyll content and the pellets for composition.

2.5. Compositional analysis

The dry matter content was determined by drying each fraction overnight at 105 °C. The protein content was determined by Dumas analysis with a NA 2100 Nitrogen and Protein Analyser (ThermoQuest-CE Instruments, Rodeno, Italy), using methionine as standard and 6.25 as a conversion factor. Although this conversion factor is high for leaf material, it was used to allow comparison to previous studies. A value based on amino acid composition could be around 5.3. Each sample was measured in