PURIFICATION AND FUNCTIONAL PROPERTIES OF POTATO PROTEIN FRACTIONS

PhD thesis by
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PREFACE

This PhD thesis summarises the results obtained by Jesper Malling Schmidt during the period from May 2013 to September 2016. The work was conducted at the Department of Food Science, Aarhus University.

A total of five scientific papers were prepared, with one being accepted, one submitted and three presently as drafts. A patent application was prepared based on a new protein purification producer. However it was decided not to proceed with the patenting procedure.

The project was funded by Future Food Innovation, KMC/AKV Langholt and the Graduate School of Science and Technology, Aarhus University.

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A special thanks to Bashar Amer and Yu Fu for their great hospitality in lending me a bed when my work in the lab extended to the wee hours of the night. Furthermore, thank you to Bashar Amer and Thao Thi Thu Le for proofreading.

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I would also like to thank my family and friends for their support during these three years.
LIST OF PUBLICATIONS INCLUDED IN THE THESIS

Paper I

Effect of membrane material on separation of proteins in potato fruit juice with special emphasis on polyphenol oxidase following ultrafiltration
Jesper Malling Schmidt, Mathias Greve-Poulsen, Henriette Damgaard, Marianne Hammershøj, Lotte Bach Larsen
Food and Bioprocess Technology, May 2016, Volume 9, Issue 5, pp 822-829

Paper II

A new two-step chromatographic procedure for fractionation of potato proteins with potato fruit juice and spray dried protein as source materials
Jesper Malling Schmidt, Mathias Greve-Poulsen, Henriette Damgaard, Anne Vuholm Sunds, Zbyněk Zdráhal, Marianne Hammershøj, Lotte Bach Larsen
Manuscript in preparation, intended for publication in Food and Bioprocess Technology

Paper III

Gel properties of potato protein isolate and purified fractions by small-scale and large deformation rheology – impact of drying method, protein concentration, pH and ionic strength
Jesper Malling Schmidt, Henriette Damgaard, Mathias Greve-Poulsen, Anne Vuholm Sunds, Lotte Bach Larsen, Marianne Hammershøj
Manuscript in preparation, intended for publication in Food Hydrocolloids

Paper IV

Foam and emulsion properties of potato protein isolate and purified fractions
Jesper Malling Schmidt, Henriette Damgaard, Mathias Greve-Poulsen, Lotte Bach Larsen, Marianne Hammershøj
Manuscript in preparation, intended for publication in Food Hydrocolloids
Paper V

Appearance and textural properties of sheared potato protein isolate gels – impact of drying method, pH and ionic strength
Jesper Malling Schmidt, Henriette Damgaard, Mathias Greve-Poulsen, Lotte Bach Larsen Marianne Hammershøj
Manuscript submitted to LWT – Food Science and Technology

Paper VI

Patent application: A method for providing several fractions of patatin and protease inhibitors
Inventors: Jesper Malling Schmidt, Marianne Hammershøj, Lotte Bach Larsen

LIST OF ABBREVIATIONS

BV Biological Value
DEAE FF Diethylaminoethanol Fast Flow
EDTA Ethylenediaminetetraacetic acid
FPLC Fast protein liquid chromatography
IEF isoelectric focusing
IEX Anion exchange chromatography
HIC Hydrophobic exchange
HPLC High-performance liquid chromatography
MALDI-TOF Matrix-assisted laser desorption-ionization time of flight
Pat Patatin
PDCAAS Protein digestibility-corrected amino acid score
PES Polyethersulfone
PI Protease inhibitor
PFJ Potato fruit juice
PPO Polyphenol Oxidase
RC Regenerated cellulose
SDS-PAGE Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
TGA Total Glycoalkaloids
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**Summary**

During industrial manufacturing of potato starch, a protein laden side-stream known as potato fruit juice (PFJ) is produced. To isolate the proteins in PFJ an acid and heat precipitation have traditionally be used, but the resulting denatured protein powder has poor functional properties and high concentrations of toxic glycoalkaloids and can therefore not be used as a food ingredient. Gentle purification methods have resulted in native potato protein with good functional properties, but problems still exists with the final protein powder, being brown and having too high polyphenol oxidase (PPO) activity and glycoalkaloid content.

A new purification protocol was developed based on an initial anion exchange (IEX) step followed by hydrophobic interaction chromatography (HIC). Liquid potato fruit juice or re-suspended spray-dried protein was separated by IEX resulting in two fractions; a protease inhibitor (PI)-rich fraction and a patatin-rich fraction. Each of these fractions were further re-chromatographed on HIC, each resulting in two new sub-fractions, a fraction of low hydrophobicity (HIC 1) and a fraction of high hydrophobicity (HIC 2).

Quality parameters like total phenol content, total glycoalkaloid content, PPO activity and colour was determined in freeze-dried fractions following purification. The total glycoalkaloid content was below the 150 ppm limit set by the EU-commission in three out of four tested HIC fractions. It was found that the glycoalkaloid α-solanine was being enriched in the PI fraction, and α-chaconine in the patatin fraction. PPO activity was significantly lower in HIC fractions compared to IEX fractions. Total phenol content was however not improved as based by results from the Folin-Ciocalteu assay. The PI fraction produced from re-suspended powder had a high lightness, while the patatin fraction was brown. For PFJ had the patatin fraction high lightness and the PI fraction was dark due to PPO catalysed browning during purification. HIC fractionation could improve the colour of patatin from PFJ but the PI fraction became darker.

An effort was made to diminish enzymatic browning early in the industrial process by selective retention of PPO by ultrafiltration. This was however not possible due to membrane fouling, but differences in retention of potato proteins were found to be depending on membrane material.

An effort was made to selectively retain PPO by ultrafiltration but this was not possible.
The IEX and HIC fractions were used for foaming and emulsion studies and it was found that the most hydrophobic fractions produced the most foam and the best emulsions. These observations could be correlated to measurements of surface- and interfacial tension.

Spray-dried and freeze-dried total protein powder and a purified patatin fraction were used for studies of gelation by oscillatory rheology and uniaxial compression with 8 and 15 % (w/w) concentrations, respectively. Spray-dried protein gave higher gel strength than freeze-dried at all tested pH and salt conditions. Gel strength was lowest at conditions that promoted low solubility and generally at the pH of ~4.7. Gels of patatin were significantly more elastic and could form transparent gels at alkaline pH in contrast to gels of total protein powder.

Spray-dried and freeze-dried protein was used for 3 % (w/w) shear gels as a possible model for a protein drink. At pH 3 a significant difference was found between drying method since freeze-dried powder led to a Newtonian liquid while spray-dried powder had a shear thinning response.

The results of this thesis provided new knowledge about functional properties of potato proteins and the impact of protein purification on final protein quality and functionality.
**Sammendrag (DK)**

Ved industriel produktion af kartoffelmel produceres en proteinholdig sidestørm kaldet kartoffel frugt juice (KFJ). Den traditionelle måde at isolere proteinerne i KFJ sker ved udfældning med syrer og opvarmning hvorved protein denatureres. Denatureret protein har ikke gode funktionelle egenskaber (oploselighed, skumning, emulgering) og har samtidigt et højt indhold af giftige glykoalkaloider, hvilket gør proteinet uegnet til fødevarer. Kartoffelprotein kan ved blide oprensningsteknikker forblive nativt og have gode funktionelle egenskaber. Der er dog stadig kvalitetsproblemer med det færdige pulver, da det er brunt, har en høj polyphenol oxidase (PPO) aktivitet og højt indhold af glykoalkaloider.

En ny oprensningsteknik blev udviklet baseret på anionbytter kromatografi (IEX) efterfulgt af hydrofob interaktions kromatografi (HIC). KFJ eller genopløst spraytørret kartoffelprotein blev separeret ved hjælp af anionbytter kromatografi til to fraktioner; en protease inhibitor (PI) fraktion og en patatin fraktion. Hver af disse fraktioner blev separeret via HIC, hvorved to nye sub-fraktioner fremkom; en fraktion med lav hydrofobicitet (HIC 1) og en fraktion med høj hydrofobicitet (HIC 2).

Kvalitetsparametre som totalt fenolindhold, totalt glykoalkaloidindhold, PPO aktivitet og farve blev bestemt på frysetørrede fraktioner efter oprensning. Det totale glykoalkaloidindhold var under den af EU-kommissionen bestemte grænse på 150 ppm i tre ud af fire af testede HIC fraktioner. Glykoalkaloidet α-solanine blev opkoncentreret i PI fraktionen og glykoalkaloidet α-chaconine i patatin fraktionen. PPO aktivitet var signifikant lavere i HIC fraktionerne i forhold til IEX fraktionerne. Totalt fenolindhold i pulveret blev dog ikke forbedret baseret på målinger bestemt ved Folin-Ciocalteu metoden. PI fraktionen oprenset fra genopløst, spraytørret protein havde en meget lys farve hvorimod patatin fraktionen var brun. PI fraktionen fra rKFJ var derimod mørk og patatin fraktionen lys. HIC oprensning kunne yderligere forbedre farven af patatin fra kartoffel frugt juice, hvorimod PI fraktionen blev mørkere.

Det blev forsøgt at mindske den enzymatiske brunfarvning tidligt i den industrielle proces ved selektiv tilbageholdelse af PPO via ultrafiltrering. Dette var dog ikke muligt på grund af tilstopning af membranerne, men en forskel i tilbageholdelsen blev observeret afhængig af membranmaterialets kemiske opbygning.
IEX og HIC fraktionerne blev brugt til studier af skum og emulsionsegenskaber og det viste sig, at de mest hydrofobe protein fraktioner kunne danne mest skum og de bedste emulsioner. Disse observationer blev korreleret til måling af overflade og interfase spænding.


Spraytørret og frysetørret protein blev brugt til frembringelse af 3 % (w/w) omrørte geler, som en mulig model for en proteindrik. Ved pH 3 var der signifikant forskel mellem prøverne, da det frysetørrede pulver gav ophav til en newtonsk væske og spraytørret pulver til en ikke-newtonsk hastighedsfortyndende væske.

Resultaterne i denne afhandling har givet yderligere indsigt i de funktionelle egenskaber af kartoffelprotein og indflydelsen af oprensning på den endelige funktionalitet og produktkvalitet.
Motivation

The world population is booming, which results in an ever increasing demand for high quality protein. This has recently led to an exploration of alternative and sustainable protein sources from e.g. algae, insects and plants in which potato presents itself as a potential source. Potato proteins are found in a side stream from industrial starch production called potato fruit juice. This side stream product is similar to whey from cheese production, previously seen as a waste material and used in animal feeding and field fertilization. To concentrate the potato protein a heat and acid precipitation method was developed and the protein used for animal feed. The quality of potato protein was not suitable for human consumption, due to the processing induced protein denaturation which affects its functional properties and increases the level of the toxic glycoalkaloids. In recent years new advanced purification techniques have been developed that permits the use of potato protein in food products. In Denmark, 9000 tons of denatured potato proteins are produced with an estimated price of 90 million DKK (12.1 mio. EUR) (Eriksen 2014). If a new advanced purification method for potato proteins can be developed this should give native protein with improved functional properties which will it result in a price premium, and a good utilization of an excellent protein source.
1 Project overview

1.1 Aims
The aims of this project are to fractionate potato proteins from either liquid potato fruit juice (PFJ) or spray-dried powder, and to evaluate the quality of the purified fractions by numerous parameters e.g. purity, colour, polyphenol oxidase activity and glycoalkaloid content. The fractions will be investigated for their functional properties e.g., gelation, foaming and emulsifying which will provide novel and applicable knowledge for the food industry. The new protein fractions are preferably required to have a white colour, low polyphenol oxidase (PPO) activity, a total glycoalkaloid content below 150 ppm and increased functional properties when compared to a spray-dried protein isolate.

Hypotheses:

- Purification of potato protein from either liquid potato fruit juice or re-suspended spray-dried potato protein powder by ion exchange and hydrophobic interaction chromatography will give a final protein powder of higher purity with lower amounts of phenolic compounds, polyphenol oxidase activity and glycoalkaloids.

- Purification obtained by hydrophobic interaction chromatography will give new protein fractions with increased foaming, emulsification and gel properties compared to ion exchange fractions.
2 General introduction

2.1 Potato
Potato plant belongs to the nightshade family (Solanaceae), which also includes the common crops capsicum, eggplant, tomato and tobacco (Friedman 2006). Potatoes have a dry matter content of ~20 %, of which 10-18 % is starch, 1-7 % sugars, 1-2 % protein, 0.5 % fibre, 0.1-0.5 % lipids and the vitamins A and C are found in trace amounts (Singh and Kaur 2016).

Potato is the fourth most important crop in the world after rice, wheat and maize and the annual production is more than 300 million tons (Singh and Kaur 2016). In Denmark 900.000 tons are produced annually which is processed to 210.000 tons of starch, 9000 tons of potato protein (as in denatured forms), 110.000 tons of potato pulp and 750.000 m³ of potato fruit juice and process water (Eriksen 2014).

2.2 Potato proteins
Potato proteins are found in PFJ after rasping of potatoes during the initial stages of potato starch production. PFJ contains 5 % (w/w) dry matter, including 35 % protein, 35 % sugars, 20 % minerals, 4 % organic acids and 6 % of other compounds such as polyphenols (Knorr et al. 1977). The protein content varies, but has been reported to be 10.1 and 11.3 g/L, for model PFJ and industrial PFJ, respectively, with a pH near 6 and a conductivity of 8-10 mS/cm (Straetkvern and Schwarz 2012). Other sources have reported higher (15.3 g/L) (Bartova and Barta 2008) or lower (3.5 g/L) values (Liu et al. 2013).

The proteins can be divided in three overall classes, patatin, protease inhibitors and others, with relative values of ~40, ~50 and ~10 %, respectively (Pouvreau et al. 2001). These values may, however, vary greatly between different potato varieties and growing season (Jorgensen et al. 2006; Barta and Bartova 2008).

2.2.1 Patatin
Patatin was named in 1980 by Racusen and Foote (1980) who purified the protein by binding to diethylaminoethanol (DEAE) and concanavalin A and found the protein to be glycosylated and have a molecular weight of 45 kDa and pI of 4.5-5.1. Later studies revealed that patatin exists as a dimer at native conditions and that the dimer could be dissociated by sodium dodecyl sulfate (SDS), but not by β-mercaptoethanol, thereby showing absence of disulfide
bonds (Racusen and Weller 1984). Patatin consists of 362 amino acid residues, and contains one free cysteine (Pots et al. 1999b).

The patatins from the potato variety Bintje were divided into four groups based on anion exchange chromatography (Table 1), with isoform A and B representing 62 and 26 % of the total amount of patatin, respectively (Pots et al. 1999a).

<table>
<thead>
<tr>
<th>Table 1 Biochemical properties of patatin isoforms from potato variety Bintje, adapted from Pots et al. (1999a). Isoform C not shown.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patatin family</td>
</tr>
<tr>
<td>SDS-PAGE</td>
</tr>
<tr>
<td>IEF-PAGE</td>
</tr>
<tr>
<td>Native PAGE</td>
</tr>
<tr>
<td>MALDI-TOF MS</td>
</tr>
</tbody>
</table>

Later studies have shown a high variation in patatin content between different potato varieties with a relative abundance between 5.4-35.4 % (Barta et al. 2012). The glycosylation patterns is also species dependent, with one, two or three glycosylations, giving molecular weights of ~40.6, 41.8 or 42.9 kDa, with relative proportions of 39.3-80.9, 4.6-48 and 0-23.7 %, respectively (Barta et al. 2012). The variety Kuras potatoes have five different possible glycosylation sites at asparagine 60, 90, 115 and 203 and at threonine 270 (Welinder and Jorgensen 2009).

Patatin has a relatively low temperature of unfolding as determined by differential scanning calorimetry of 59-60 °C (Creusot et al. 2011). At 20 °C patatin has a compact structure with 33 % α-helix and 46 % β-sheet, upon heating, α-helix unfolds from 45-55 °C, and unfolding of β-sheet from 50-90 °C. Cooling causes refolding with a 30 % α-helix and 26 % β-sheet content at 20 °C. The tertiary structure is already affected by temperatures exceeding 28 °C (Pots et al. 1998). Upon heating aggregates are formed with molecular weights as assessed by SDS Polyacrylamide gel electrophoresis (SDS-PAGE) of 43, 82 and 108 kDa, respectively. These masses indicate a monomer, dimer and a trimeric structure with altered mobility. These aggregates dissociate to 43 kDa bands in the presence of β-mercaptoethanol, suggesting the presence of an intermolecular disulfide bond, involving the cysteine residue. Heating in the presence of N-ethylmaleimide, which inhibits disulphide bond formation, also produced bands at 43, 82 and 108 kDa, and it was suggested that disulfide bond formation was not a determining factor of patatin aggregation (Pots et al. 1999b). Patatin can be heat treated an
still remain in solution if the ionic strength is kept low (\(< 15 \text{ mM}\) (van Koningsveld et al. 2001). Changes in pH can also unfold patatin, with loss in secondary and tertiary structure below pH 4 (Pots et al. 1998).

The solubility of patatin is shown in Figure 1. Patatin has a solubility minimum around pH 3.5, when ionic strength is 200 mM, while at lower ionic strength of 15 mM the solubility minimum is at pH 4.5.

![Figure 1 Solubility of PFJ (□), (NH₄)₂SO₄ precipitated PFJ (●), PI (▽), patatin (▲) at ionic strength 200 mM A), and ionic strength 15 mM B). Adapted from (van Koningsveld et al. 2001).](image)

Patatin has different enzymatic activities with the most prominent being lipid acyl hydrolase. Patatin has broad specificity toward phospholipids, mono- and diglycerides, glycolipids, with a higher activity for monoglycerides than diglycerides (Anderson et al. 2002). The activities towards triglycerides have been investigated with highest activity on short chain fatty acids with chain length of four, six and eight carbons, compared to chain length of ten to eighteen (Spelbrink et al. 2015). This activity have been exploited in production of cheese with additional flavour due to fatty acid release (Spelbrink et al. 2015), and synthesis of monoglycerides from free fatty acids and glycerol (Macrae et al. 1998).

### 2.2.2 Protease inhibitors

The protease inhibitors represents are more heterologous group of potato proteins, with the majority of them having a molecular weight below 40 kDa, and having pI in the range of 5.7-9.0 (Table 2). The protease inhibitors are generally highly soluble proteins with higher solubility than patatin (Figure 1).
As the name implies has these proteins protease inhibitor activities towards many different proteases e.g. trypsin, chymotrypsin, papain, cathepsin D with potato serine protease inhibitor showing the highest inhibition of trypsin/chymotrypsin, PCPI being the only inhibitor of papain, and PAPI the only inhibitor of cathepsin D (Pouvreau et al. 2001).

**Table 2** Relative proportions of potato protein inhibitors in PFJ with indication of relative content, molecular mass, number of subunits and pI, from (Alting and Pouvreau 2011). A (van den Broek et al. 2004). B (Rodis and Hoff 1984).

<table>
<thead>
<tr>
<th>Name</th>
<th>% in PFJ</th>
<th>Molecular weight in kDa (number of sub-units)</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Potato inhibitor 1 (PI-1)</td>
<td>5</td>
<td>40 (5) or (6)A</td>
<td>5.7-7.8</td>
</tr>
<tr>
<td>Potato serine protease inhibitor (PSPI/PI-2)</td>
<td>22</td>
<td>20-21 (2)</td>
<td>5.8-6.9</td>
</tr>
<tr>
<td>Potato cysteine protease inhibitor (PCPI)</td>
<td>12</td>
<td>20-23 (1)</td>
<td>5.8-9.0</td>
</tr>
<tr>
<td>Potato aspartyl protease inhibitor (PAPI)</td>
<td>6</td>
<td>20-22 (1)</td>
<td>6.2-8.7</td>
</tr>
<tr>
<td>Potato Kunitz-type protease inhibitor (PKPI)</td>
<td>4</td>
<td>20 (1)</td>
<td>8.0-9.0</td>
</tr>
<tr>
<td>Potato carboxypeptidase protease inhibitor (PCI)</td>
<td>1</td>
<td>4.3 (1)</td>
<td>?</td>
</tr>
<tr>
<td>Potato multicystatin B</td>
<td>?</td>
<td>89 (pH 4.3), ~340 (pH 8.4)</td>
<td>?</td>
</tr>
<tr>
<td>“Other serine protease inhibitors (OSPI)”</td>
<td>2</td>
<td>21-22 (1,2)</td>
<td>7.5-8.8</td>
</tr>
</tbody>
</table>

Detailed structural studies have been conducted with PSPI and PCPI. PSPI is a dimeric protein with disulphide-linked subunits of 16.1 and 4.1 kDa. Depending on isoform is the denaturation temperature ($T_m$) 62.2-65.9 °C. The heat stability of the protein is enhanced by the disulphide bond since heating in presence of reducing compounds lowers the denaturation temperature to 45.2 °C. Upon heating is a 80 kDa aggregate formed which represents four PSPI proteins. Non-reducing SDS-PAGE results in a band at 80 kDa, while reducing conditions cause dissociation to two bands at 15 and 6 kDa, indicating the aggregate is linked by disulphide interchange (Pouvreau et al. 2005a).

PCPI consists of a single subunit with two internal disulphide bridges. The protein unfolds at ~67 °C and form aggregates with sizes exceeding 100 kDa but no aggregates are formed when heating is conducted in reducing conditions (Pouvreau et al. 2005b).

Potato inhibitor 1 have been expressed in yeast and the protein have shown high heat stability with denaturation at ~88 °C (van den Broek et al. 2004). The small Potato carboxypeptidase protease inhibitor have also shown high heat stability, retaining solubility at temperature of 70 °C (Bartova and Barta 2008).
The potato multicystatin is a special inhibitor with eight inhibitory domains and a size of 85-89kDa. This protein forms crystals at neutral or alkaline pH and is soluble at pH 5 or below. Digestion of the monomer with trypsin releases five 10 kDa and one 35 kDa peptides with functional inhibitory domains (Green et al. 2013).

### 2.2.3 Modifications of patatin and protease inhibitors

The potato proteins have been modified by a number of techniques to enhance the functional properties.

van Koningsveld et al. (2002c) performed limited denaturation by either heating protein solutions (80 °C, 10 min), or by precipitation with ethanol. This led improved foaming for patatin and PI compared to control conditions. Patatin was also denatured by pH adjustment to pH 3 following readjustment to pH 7, which also gave increased foaming compared to control conditions.

Patatin have been succinylated, which introduced additional negative surface charge and caused slight unfolding. Emulsions made with succinylated patatin had increased emulsion stability at high ionic strength conditions, this was ascribed to the partial unfolding and hereby a higher adsorption to the oil-water interface (Delahaije et al. 2014b).

Patatin have been glycosylated with glucose, maltotriose and maltopentatose and it was found that modification with maltotri- and pentatose increased emulsion stability at the isoelectric point due to steric repulsion (Delahaije et al. 2013). Seo et al. (2014) performed glycosylation with galactose, galactooligosaccharides and galactan and found that modification with galactooligosaccharides led to a more heat stable patatin with unfolding at 70-90 °C compared to 50-70 °C for control, furthermore did galactose modified patatin have increased emulsion stability at pH 3 and galactose and galactan had smaller emulsion droplet size at pH 7 than unmodified patatin.

Potato protein concentrated by ultrafiltration has been acetylated, resulting in increased solubility, higher water- and oil binding capacity and emulsion stability but decreased foam overrun and stability (Miedzianka et al. 2012).

Baier and Knorr (2015) have recently modified the structure of a potato protein isolate or purified patatin by high pressure (200, 400 and 600 MPa). High pressure resulted in slightly
elevated hydrophobicity (1.3 fold), formation of protein complexes from patatin with molecular weight between 100-150 kDa as determined by gel electrophoresis, and 177 % increased foam stability compared to control samples.

Potato proteins have also been hydrolyzed with proteases. A commercial heat and acid precipitated potato protein concentrate was treated with alcalase resulting in significantly better solubility, significantly higher oil holding capacity, significantly higher foam overrun and stability and increased lightness ($L^*$) of freeze dried hydrolysate (Miedzianka et al. 2014)

2.2.4 Other proteins

Many other proteins are present in potato, but in lower amounts compared to patatin and the protease inhibitors. A number of enzymes are associated with starch synthesis e.g. starch phosphorylase L-1, α-Glucan water dikinase or 4-α-glucanotransferase (Jorgensen et al. 2006) Oxidative enzymes like peroxidase or lipoxygenase are also present, with lipoxygenase representing 10 % of the total protein in the cultivar Kuras, that’s used in Denmark (Jorgensen et al. 2006). Lipoxygenase catalyzes oxidation of polyunsaturated fatty acids to reactive hydroperoxides. Industrially the enzyme can be used for bleaching of various pigments or as an enhancer of dough structure in bread making (Heshof et al. 2016). A potential negative effect of this protein is production of volatile compounds associated with off-flavour or odour (Murat et al. 2013; Robinson et al. 1995), which could impact the usability of potato protein.

2.3 Nutritional aspects of potato proteins

Potato proteins have excellent amino acid composition in relation to nutrition, and high digestibility. When compared to other common protein sources the potato proteins have high contents of lysine, phenylalanine and tyrosine and therefore essential amino acids, especially compared to soy protein (Table 3). The Biological Value and PDCAAS are also very good and better than a range of other plant derived proteins (Table 4).

<table>
<thead>
<tr>
<th>Table 3 Amino acid content in a selection of common protein sources, from (Alting and Pouvreau 2011).</th>
</tr>
</thead>
<tbody>
<tr>
<td>g/100 g protein</td>
</tr>
<tr>
<td>protein</td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Branched AA</td>
</tr>
<tr>
<td>Essential AA</td>
</tr>
<tr>
<td>Met + Cys</td>
</tr>
<tr>
<td>Phe + Tyr</td>
</tr>
</tbody>
</table>
Table 4 Protein Digestibility Corrected Amino Acid Score (PDCAAS) and Biological Value (BV) of a selection of protein sources, from (Alting and Pouvreau 2011).

<table>
<thead>
<tr>
<th>Protein Source</th>
<th>PDCAAS</th>
<th>BV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole egg</td>
<td>1.19</td>
<td>1.0</td>
</tr>
<tr>
<td>Casein</td>
<td>1.23</td>
<td>0.88</td>
</tr>
<tr>
<td>Soy protein</td>
<td>0.91</td>
<td>0.77-0.84</td>
</tr>
<tr>
<td>Wheat protein</td>
<td>0.43</td>
<td>0.59</td>
</tr>
<tr>
<td>Commercial patatin</td>
<td>0.99</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Both patatin and PI proteins have been in vivo digestibility tested against whey or casein. It was shown that the release of branched amino acids over time was lowest for PI proteins, followed by patatin, casein and whey. The postprandial insulin response was not elevated after intake of patatin or PI, in contrast did casein induce a moderate increase and whey a significantly higher insulin response (He et al. 2013).

An interesting observation has been made upon feeding rats with potato protein inhibitors. Pre-feeding with PI resulted in a significantly lower food intake compared to control, furthermore gastric emptying was delayed and the plasma hormone cholecystokinin increased. Cholecystokinin levels are normally increased after a meal as a signal for satiety (Komarnytsky et al. 2011). Similar results have been observed with concentrated PFJ (Chen et al. 2012).

Potato proteins are generally seen being low in causing allergic reactions. Of ~800 tested infants 15 % showed reaction to eggs, 9 % to cow’s milk and only 5 % to potato (Majamaa et al. 2001). Off the potato proteins patatin is seen as the protein causing allergic responses. Heating can reduce allergenicity of patatin and it have been shown that aggregation is the cause of this, with a 25-fold reduction in affinity between Immunoglobulin E and patatin if patatin aggregates alone, or 110-fold if patatin is aggregated in presence of other potato proteins (Koppelman et al. 2002).

2.4 Phenolic compounds and Polyphenol Oxidase
The polyphenol chlorogenic acid represents 90 % of the polyphenols in potatoes and contributes to the health promoting effect of potatoes due to its antioxidant activity (Friedman 1997). Polyphenols and the enzyme PPO is however unwanted during purification of potato protein due to a number of chemical reactions that affect the quality of the final protein powder.
Potato PPO has a molecular weight of 40-69 kDa (Hunt et al. 1993; Marri et al. 2003; Eidhin et al. 2010) and a reported native multimeric structure with a mass of 340 kDa (Marri et al. 2003). PPO catalyzes the hydroxylation of mono-phenols to o-diphenols and oxidation of o-diphenols to colored o-quinones (Figure 3) (Ramírez et al. 2003). The presence of reducing compounds like ascorbic acid can reduce quinones back to diphenols (Ramírez et al. 2003). Further reaction between quinones results in development of dark brown or black melanin complexes that cannot be separated from potato proteins by ultrafiltration (Friedman 1997; Straetkvern and Schwarz 2012).

![Chemical structure of the most important polyphenol in potatoes, chlorogenic acid, from (Rawel et al. 2002).](image)

**Figure 2** Chemical structure of the most important polyphenol in potatoes, chlorogenic acid, from (Rawel et al. 2002).

The phenolic compounds can bind proteins by different mechanisms and experiments with canola proteins at pH 12 indicated ~43 % free phenolics, 30 % bound by ionic interactions, ~8 % hydrophobic, 5 % by hydrogen bonding and 5 % covalently bound. At pH 3.5 ~58 % of the phenolics were free and roughly 10 % bound by ionic interactions (Xu and Diosady 2000). Covalent interaction to amino acids and proteins can happen at alkaline pH or in the presence of PPO, these interactions results in loss of essential amino acids, change in isoelectric point to lower pH values, decreased protein solubility, decreased or increased surface hydrophobicity, increase denaturation temperature, altered digestion rate and cross-linking of proteins (Kroll and Rawel 2001; Rawel et al. 2002; Prigent et al. 2007; Eidhin et al. 2010).
PPO can be inhibited by heat treatment (80 °C for 15 min), low pH (pH < 3.5) or by addition of antioxidants or inhibitors, like ascorbic acid or sodium metabisulfite (Eidhin et al. 2010).

### 2.5 Glycoalkaloids

Plants in the nightshade family produce glycoalkaloids as secondary metabolites. Glycoalkaloids are toxic to pests of the potato but also to humans, and ingestion of high amounts of glycoalkaloids leads to nausea, diarrhoea, fever and even death. Consumption of green potatoes should be avoided since green potatoes has considerably high levels of glycoalkaloid; furthermore, potatoes with high glycoalkaloid content has a bitter taste and can produce a burning sensation in the mouth (Friedman 2006). Glycoalkaloids are found in the highest concentration in potato flowers, leaves and fruits and in the tuber is the highest concentration found in the skin (Friedman 2006). Due to toxicity a limit of 150 ppm total glycoalkaloids (TGA) in the final protein powder has been set by the EU-commission (Byrne 2002)

Glycolakaloids consists of a hydrophobic steroidal (aglycone unit) and a hydrophilic trisaccharide (Glycosidic unit) (Figure 4). In potatoes α-solanine and α-chaconine are the two most abundant glycoalkaloids. They share the same aglycone unit (solanidine) but differ in the glycosidic unit.

![Figure 4](image.jpg)

**Figure 4** Chemical structure of α-solanine with display of aglycone and glycosidic units. From (Milner et al. 2011).

It has been found that upon mixing α-solanine with soil samples the glycoalkaloid would be degraded with a half-live of the process of 1.8-1.9 days. One possible explanation for this degradation was due to microorganisms (Jensen et al. 2009). Endogenic enzymes present in the potato can also degrade glycoalkaloids in a sequential manner leading to the aglycone
Extracts from potato sprouts or haulm showed a degree of hydrolysis of ~80 % after 96 hours of incubation (Nikolic et al. 2006).

Relatively few studies concerning the removal of glycoalkaloids or the interaction between protein and glycoalkaloids have been reported. Glycoalkaloids have been removed from potato juice by Adsorptive Bubble Separation, a technique relying on selective adsorption of molecules to the foam interface with collection of the wanted molecules in collapsed foam. Studies at pH 5, 6, 7 and 8 were conducted with a 99.5 % recovery at pH 6, without the potato proteins being concentration (Backleh et al. 2004). A patented method have been made to lower glycoalkaloid content in acid/heat precipitated proteins. Here the powder is suspended in water followed by acidification to pH 3 and washing/extraction for at least 30 minutes, then dehydration and washed in water (Kemme-Kroonsberg et al. 2000). Another patented method relies on adsorption of glycolakaloids to activated carbon at pH 7 for patatin or pH 3 for PI which essentially removes all glycoalkaloids (Giuseppin and Spelbrink 2009)
3 Purification techniques

In this section some of the many methods used to purify potato proteins are discussed.

3.1 Precipitation and complexation

Precipitation by heat and acid has historically been the method of choice to isolate proteins from PFJ (Strolle et al. 1973; Knorr 1977). The method gives a high recovery, but the resulting protein powder is not fit for food applications due to a very low resolubility (Table 5) and a salty bitter taste (Zwijnenberg et al. 2002) and high amounts of glycoalkaloids (Lokra et al. 2008). Acids alone can also precipitate the potato proteins by lowering pH below 5. The recovery is lower, but resolubility higher with values up to ~27 % (Table 5). Proteins have minimum solubility at the isoelectric point, but precipitation of potato protein in PFJ behaves differently with maximum precipitation at pH 3 which is lower than pI of both patatin (4.6-5.2) and the protease inhibitors (5.7-9.0), furthermore dialysis of PFJ shifts the precipitation-optimum to pH 3.5-4 and increased both recovery and resulubility (Table 5). It is was hypothesised that the difference between PFJ and dialysed PFJ was due to removal of phenolic compounds known to react with proteins (van Koningsveld et al. 2002b)

Salts like Ammonium sulfate (NH$_4$)$_2$SO$_4$ will decrease the solubility of proteins, also known as “salting-out” when added in sufficient amounts. High salt concentration will promote conformational changes and increase hydrophobic interactions between proteins resulting in precipitation (Wingfield 2001). (NH$_4$)$_2$SO$_4$ precipitation is seen as a mild method of precipitation (van Koningsveld et al. 2002c) and the resolubility is also high with a values of ~72 % (Table 5).

Metal salts interact with proteins by forming complexes between specific amino acids (Histidine, Tryptophan and Cysteine) and the metal ions (Zachariou and Hearn 1996; van Koningsveld et al. 2002b). FeCl$_3$ precipitation gives both high yields and good resolubility and precipitation with FeCl$_3$ can significantly reduce the content of glycoalkaloids compared to conventional heat/acid precipitation (Bartova and Barta 2009), furthermore metal salts can form complexes with polyphenols (McDonald et al. 1996) and this may lower the possibility of protein-phenol interactions.
Table 5 Examples of purification techniques relying on precipitation or complexation.

<table>
<thead>
<tr>
<th>Principle</th>
<th>Conditions and outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Heat and acid</strong></td>
<td>¹PFJ adjusted to 5.8-6.2 by HCl, steam injection at 104 °C, recovery 99 %. ²PFJ adjusted with H2SO4 to pH 4.8, incubation 2 min. at 100 °C, recovery 90.2 %, resolubility (pH 7) &lt; 5 %.(Strolle et al. 1973), ²(Waglay et al. 2014), ³(Waglay et al. 2014), ³(Barta et al. 2008), ³(van Koningsveld et al. 2002b)</td>
<td></td>
</tr>
<tr>
<td>Acid</td>
<td>¹PFJ adjusted with H2SO4 to pH 2.5 at 25 °C, recovery 64.7 %, resolubility (pH 7) ~12 %. ²PFJ adjusted to pH 3.5 with HCl, H2SO4, acetic acid, citric acid at 0 or 22 °C, recovery was highest at 22 °C with values from 41.5 % (H2SO4) to 54.5 % (acetic acid), resolubility (pH 7) 0 % (acetic acid) to 4 % (HCl). ³PFJ and dialysed PFJ adjusted to pH 4.0 with H2SO4, recovery ~47 % and 90 %, resolubility (pH 7) <del>10 % and</del>60 %, respectively. ³PFJ adjusted to pH 3.5 with citric acid, recovery ~52 %, resolubility (pH 7) ~27 %. ¹(Waglay et al. 2014), ²(Barta et al. 2008), ³(van Koningsveld et al. 2002b)</td>
<td></td>
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<tr>
<td>Inorganic salt</td>
<td>¹(NH4)2SO4 (60 % saturation) at 4 °C, recovery 98.6 %, resolubility (pH 7) ~72 %. ¹FeCl3 (20 or 40 mM) or MnCl2 (final concentration 20 mM) addition to PFJ at pH 5 at 25 °C, recovery 57.4, 75.2 % and 16.8 % respectively, resolubility (pH 7) of FeCl3 (20 mM) ~48 % (Other conditions not determined). ²FeSO4, FeCl3, ZnCl2 (final concentration 15 mM) added to PFJ at 0 or 22 °C, recovery was lowest for ZnCl2 at 0 °C (25.8 %) and highest for FeCl3 at 0 °C (86.4 %), resolubility (pH 7) from 18.5 % (FeSO4, 22 °C) to 79.3 % (FeCl3, 0 °C). ¹(Waglay et al. 2014), ²(Barta et al. 2008)</td>
<td></td>
</tr>
<tr>
<td>Organic solvent</td>
<td>Ethanol addition (20 % v/v) to PFJ 4 °C, recovery 55.2 %, resolubility (pH 7) ~35 %. ²Addition (20 % w/w) of ethanol, methanol, acetone or 2-propanol to PFJ (pH 5) 1 h at 0 or 22 °C, recovery was generally higher at 0 °C with values of 25 % (acetone) to 64.5 % (2-propanol), resolubility (pH 7) was from 5.9 % (acetone) to 49.8 % (ethanol, methanol) when precipitated at 0 °C and 1.1 % (acetone) to 8.2 % (methanol) at 20 °C. ³Ethanol addition (30 % v/v) to PFJ at 0 °C (pH 5), recovery ~90 %, resolubility (pH 7) ~80 %. ¹(Waglay et al. 2014), ²(Barta et al. 2008), ³(van Koningsveld et al. 2002b)</td>
<td></td>
</tr>
<tr>
<td>Complexation</td>
<td>¹Carboxymethyl cellulose (CMC) was added to PFJ (pH 2.5) at a ratio of CMC to protein of 0.3 at 25 °C, recovery 75.3 %, resolubility (pH 7) ~9 %. ²Solutions of 0.2 % (w/v) of chitosan, 1-carrageenan, xanthan gum, sodium alginate, gum arabic or CMC was added to PFJ at either pH 3 (chitosan) or pH 6 and precipitates collected by centrifugation after additional pH adjustment, recovery ~52 %, ~100 %, ~99 %, ~95 %, ~74 % and 85 %, respectively. ¹(Waglay et al. 2014), ²(Kong et al. 2015)</td>
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</table>

Organic solvents promote precipitation by disrupting protein-protein interactions in native protein and induce reorganization of the secondary structure ultimately leading to denaturation of proteins (Yoshikawa et al. 2012). Depending on temperature and solvent type high recovery and resolubility is possible with values of ~90 % and ~80 % respectively (Table 5). Ethanol precipitation have been found to have a larger impact on changes in secondary and tertiary structure of patatin than on the PI proteins (van Koningsveld et al. 2002a), but
patatin still maintains its enzymatic activity after ethanol precipitation (Bartova and Barta 2009). Precipitation with ethanol has also been found to significantly reduce the content of glycoalkaloids compared to heat/acid precipitation (Bartova and Barta 2009).

Complexation gives precipitation by electrostatic interactions between protein and polysaccharide at pH values that promote opposing surface charge (Kong et al. 2015). CMC complexation can result in high recovery but a resolubility of less than 10 % (Table 5). (Kong et al. 2015) showed recoveries of 99 and 100 % when using carrageenan and xanthan respectively, but unfortunately no data on resolubility was applied.

It should be noted that the different methods of precipitation, not only differ in yield and degree of resolubility, but the precipitate also varies in relative composition between patatin and PI proteins, e.g. has chitosan high affinity for patatin while carrageenan binds all potato proteins (Kong et al. 2015). 30 % ethanol results in a precipitate with 49 % patatin, 20 mM FeCl₃ in 33.2 % and 20 mM MnCl₂ in 20.4 % (Waglay et al. 2014).

Of the methods listed above ethanol seem promising with both high yield and high recovery, but to achieve this precipitation has to be done at 0 °C. FeCl₃ is also promising with up to 70 % resolubility when performed at 22 °C, one drawback might be the resolubility is affected if EDTA is left out, as noted in (Bartova and Barta 2009).

3.2 Chromatography

Chromatography can in contrast to most forms of precipitation result in good separation of patatin and protease inhibitors. Ion exchange and expanded bed adsorption (EBA) are the two mostly used techniques, with ion exchange separating proteins based on oppositely charged surfaces between protein and ligand material, and for EBA, special materials displaying both ionic and hydrophobic materials have been used (Straetkvern and Schwarz 2012.) Expanded bed adsorption is superior to conventional packed bed chromatography for a number of reasons. The crude PFJ is a particle laden liquid that may clog conventional packed bed systems unless PFJ has been e.g. centrifuged, prefiltrated or flocculate. These steps are not needed for EBA, due to fluidization of the beads, and thus no clogging (Lokra and Straetkvern 2009). EBA has been used to capture all proteins or bind patatin or PI depending on binding pH and packing material (Lokra et al. 2009; Straetkvern et al. 1999). EBA have been reported to lower PPO activity in the final protein powder, and separate brown polyphenol complexes
and glycoalkaloids from the proteins (Lokra et al. 2008; Lokra et al. 2009) hence providing a superior end product.

In an effort to optimize the conventional batch wise chromatography process a simulated moving bed setup have been used for isolation of proteins from PFJ. This method which utilises multiple columns can run in a continuous manner and by coupling this process to a ultrafiltration system have high protein recovery been possible with low buffer consumption (Andersson et al. 2008).

**Table 6 Examples of purification techniques relying on chromatography.**

<table>
<thead>
<tr>
<th>Principle</th>
<th>Conditions and outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ion exchange</td>
<td>¹PFJ (pH 7) was passed through Sephadex G-50 and then onto a DEAE-cellulose column with binding of patatin and flow through of PI proteins. ²PFJ (pH 8) applied to DEAE-Sepharose with binding of patatin, elution with 0.5 M NaCl. Unbound fraction applied to SP-Sepharose with binding of PI, elution with 1 M NaCl, recovery ~75 %.</td>
<td>¹(Racusen and Foote 1980), ²(Ralet and Gueguen 2000).</td>
</tr>
<tr>
<td>Expanded bed adsorption</td>
<td>¹PFJ (pH 4.5) loaded onto EBA column with a mixed mode resin binding all proteins, elution 20 mM NaOH, recovery 54 % protein, TGA content 286 ± 95 ppm, Lightness (CIE) 73.8. ²PFJ (pH 5) applied to a EBA column with Amberlite XAD7HP resin, flow through with patatin and binding of PI, elution by addition of 20 mM NaOH, TGA content 165-174 ppm, Lightness (Hunter Lab) of powder 86 (spray dried) and 48 (vacuum freeze dried).</td>
<td>(Straetkvern and Schwarz 2012), (Zeng et al. 2013).</td>
</tr>
<tr>
<td>Simulated moving bed</td>
<td>PFJ was subjected to a 10 column simulated moving bed set-up with columns filled with Q Sepharose FF resin, both PI and patatin were bound at pH 6.5 and eluted by a high salt buffer (pH 6.5, 2 M NaCl), recovery ~80 %.</td>
<td>(Andersson et al. 2008)</td>
</tr>
<tr>
<td>Affinity</td>
<td>¹Patatin can be selectively bound by concanavalin A resins as a final polishing step, binding at pH 7 and elution by 20mM a-methyl-D-glucoside pH 7.</td>
<td>¹(Racusen 1989), also (Bohac 1991).</td>
</tr>
<tr>
<td>Hydrophobic interaction</td>
<td>¹HIC have been used as polishing instead of concanavalin A by binding to Octyl Sepharose at 0.25 M NaCl pH 7 and eluted with 50 % ethylene glycol resulting in one patatin fraction. ²Polishing by Octyl Sepharose with binding at 1 M (NH₄)₂SO₄ pH 7 and elution with H₂O.</td>
<td>¹(Racusen 1989), ²(Partington and Bolwell 1996)</td>
</tr>
</tbody>
</table>

### 3.3 Membrane filtration

Cross flow ultrafiltration is a pressure driven method where macromolecules are retained by a semi permeable membrane based on molecular weight. Compared to EBA can ultrafiltration not separate polyphenols or brown polyphenol complexes from proteins thus giving a powder with a final brown hue and higher content of chlorogenic acid (Straetkvern and Schwarz 2012). One problem often encountered with membrane concentration of PFJ is low flux due to membrane fouling. This will lead to longer processing time and probably a lower lifetime of the membrane (Zwijnenberg et al. 2002).
Adsorptive membranes have been developed which relies on ion exchange capabilities to bind proteins instead of retention by molecular weight. The ion exchange ligands are bound to an open membrane which allows high flow and loading of more crude liquids (Schoenbeck et al. 2013). These systems may provide some of the same benefits as EBA, i.e. loading of crude PFJ and binding of proteins following removal of phenolic compounds.

**Table 7** Examples of purification techniques relying on ultrafiltration.

<table>
<thead>
<tr>
<th>Principle</th>
<th>Conditions and outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-flow ultrafiltration</td>
<td>¹PFJ was concentrated and diafiltered by a 10 kDa cut-off polyether sulfone membrane; recovery 47.9 % protein, TGA 213 ± 100 ppm, Lightness (CIE) 41.3. ²PFJ was concentrated and diafiltered by various membranes (polyether sulfone, polyvinylidenefluoride, regenerated cellulose) with cut-offs of 5 to 150 kDa.</td>
<td>¹(Straetkvern and Schwarz 2012), ²(Zwijnenberg et al. 2002)</td>
</tr>
<tr>
<td>Adsorptive membranes</td>
<td>Regenerated cellulose membranes modified with either quaternary ammonium (Q) or sulfonic acid (S) ligands giving ion exchange capability. Diluted PFJ with water (1:5) was applied to membranes and elution conducted with 0.5 M NaCl pH 7 buffer, with binding of patatin to Q-membranes, and PI to S-membranes.</td>
<td>(Schoenbeck et al. 2013)</td>
</tr>
</tbody>
</table>

### 3.4 Other techniques

**Table 8** Examples of purification methods relying on principles, other than described above.

<table>
<thead>
<tr>
<th>Principle</th>
<th>Conditions and outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sieving, air classification</td>
<td>Spray drying of homogenized potato pulp at an inlet temperature of 180-235 °C and outlet of 90-110 °C followed by sieving and separation by cyclone and air classifier yielding three fractions: fibre (14 kg), starch (69 kg) and protein (17 kg) of 100 kg spray dried solids.</td>
<td>(Holm 1980)</td>
</tr>
<tr>
<td>Ion exchange</td>
<td>PFJ diluted 1:1 (pH 8.5) applied to a column with clay minerals, patatin in flow through, with elution of PI by increase in pH to 12, reduction in α-solanine by &gt; 90 % of initial value (74 ppm).</td>
<td>(Ralla et al. 2012)</td>
</tr>
<tr>
<td>Foam separation</td>
<td>PFJ adjusted to pH 7 was fractionated by two-stage foam separation. Step 1: temp. 45 °C airflow rate 100mL/min. Step 2: temp 20 °C airflow rate 250 mL/min. Recovery 73-4 % protein.</td>
<td>(Liu et al. 2013)</td>
</tr>
<tr>
<td>Enzymatic</td>
<td>Potato pulp was mixed with α-amylase to remove starch, polygalacturonase and endo-β-1.4-galactanase was added and incubated at ~43 °C for ~20 h to degrade cell walls. Filtration with a 1.2 µm filter led to a supernatant with proteins. Recovery 59.5-67 % protein.</td>
<td>(Waglay et al. 2016)</td>
</tr>
</tbody>
</table>

A number of methods which relies on other principles or less common materials are shown in Table 8. Holm (1980) showed that homogenized potato protein slurry could be spray dried and hereafter separated into protein, starch and fibre by air classification. The resulting protein was reported to have good emulsion properties (Holm and Eriksen 1980). Ralla et al. [reference]
(2012) showed that clay minerals could be used as ion exchange materials with separation of patatin and PI with a high reduction in glycoalkaloids. Liu et al. (2013) explored a technique where foam was made of PFJ and the collapsed foam collected, hereby concentrating the surface active proteins. This process seems simple but large scale systems are not common (Burghoff 2012). Waglay et al. (2016) developed an enzymatic approach where potato pulp (not PFJ) was treated with a number of enzymes, degrading starch and cell wall material, resulting in a liquid where protein was recovered after a filtration step. This method could be used in addition to the methods that uses PFJ as source material.

In summary, many methods have been utilized to concentrate the proteins from PFJ. The most promising methods seem to be precipitation with FeCl$_3$ or ethanol, but if a white product is needed EBA or the new Adsorptive membranes seem to be the optimal choice.
4 Functional properties of protein

4.1 Gelation

A gel is formed when a solution goes from liquid to solid-like state by forming a three-dimensional network, i.e. a continuous phase that is spanning the entire volume, and is embedded in a discontinuous phase, i.e. water or other liquid (Clark, 1992). The microstructure of the network will determine macroscopic properties like transparency, strength and elasticity. Protein gelation of globular proteins requires a gel-inducing agent (enzyme, salt, pH-change etc.) or process (heat, pressure, etc), and for food proteins it most often involves a heating step were the native conformation of the protein partially unfolds to a “reactive” molecule. The “reactive” molecule will form a gel if the protein is present in sufficient concentration and at pH and ionic strength conditions that favour aggregation as indicated in Figure 5 (Broersen et al. 2006).

![Figure 5](image_url) Model of gelation of a globular protein and effect of pH and ionic strength on gel-network structure. Adapted from (Doi 1993).

It is commonly stated that during protein denaturation buried hydrophobic patches and cysteines will be exposed leading to aggregation (Broersen et al. 2006). Hence, the molecular interactions forming the protein gel network can be covalent, disulphide bonds, hydrophobic interactions, electrostatic interactions, hydrogen bonds and van der Waals forces. The denaturation temperature varies between proteins depending on protein structure and the presence of internal disulphide bonds that increases conformational stability. External factors
like protein concentration, pH, salt and heating rate all affects the denaturation temperature (Creusot et al. 2011; Stading and Hermansson 1990; van Koningsveld et al. 2001).

The morphology of the aggregates depend on ionic strength and pH, with “fine-stranded” networks formed, when the pH is far from pI and at low ionic strength, while “particulate” networks are formed, when pH is near pI and at high ionic strength (Doi 1993). A special case is gels formed in two steps with the first being a heating step at conditions that don’t favour aggregation, resulting in denatured proteins that maintain solubility. The second step is introduction of salt or changes in pH that favour protein interactions, gelation will then take place either without needed heating or with the need of a second heating step (Figure 5) (Doi 1993; Alting et al. 2004).

The microstructure of fine-stranded gels is different below and above pI with stiff and short strands observed at low pH and long flexible strands at high pH (Stading et al. 1995). The different microstructures result in different macroscopic properties as well, with brittle gels at low pH and ductile at high pH (Doi 1993).

In order to evaluate both the gelation process and the resulting gel properties, rheology analysis are most often applied. The techniques can be divided in two: Small-deformation (oscillatory) studies, where measurements are conducted with so small deformation that the structure of the material stays unaltered, and large-deformation textural studies, where the structure may be irreversible destroyed. In oscillatory rheology, a sinusoidal strain or stress is applied to a material and the corresponding strain or stress measured. The material is evaluated by comparison of the phase shift and the amplitude of the stress-strain response. A viscous Newtonian fluid is out of phase and has a low amplitude, a Hookean solid is in phase and has a large amplitude while a viscoelastic material will be within these two extremes (Figure 6A). The difference in phase shift and amplitude can be used to calculate the storage modulus (G’) and the loss modulus (G’’). When G’’ is larger than G’ the material is considered to be a fluid, and when G’ is largest the material is considered to be a solid (Steffe 1996), and in rheology terms, gels are showing overall solid characteristics (Clark, 1992). G’ and G’’ can be used to calculate the phase angle tan(δ), which varies from 0-90°. The gel point can be determined by oscillatory rheology as a rapid increase in G’ or the cross-over between G’’ and G’ which translates to the point where the phase angle is 45° (Stading and Hermansson 1990).
Large deformation studies are often applied in tension or compression and gives information about the strength and the deformability of the tested material based on the measured force-deformation curve and calculations of stress and strain (Figure 6B).

Gel texture has been evaluated for particulate and fine-stranded gels. The storage modulus measured my oscillatory rheology was found to be higher for particulate compared to fine-stranded gels (Stading et al. 1995; Renkema et al. 2000). Large deformation studies have however shown the highest gel strength to be at pH = pI and lowest gel strength at pH = pI (Doi 1993; Stading et al. 1995).

Gel strength and stiffness is dependent on the ionic strength and often increase until an optimum is reached, with an increase in ionic strength from this point decreasing gel strength and stiffness (Doi 1993; Urbonaite et al. 2016).

Another important property of food gels is the ability to bind and hold water, which often is analysed as the water holding capacity. The water holding capacity is affected by gel network type with a fine-stranded network having higher water-holding capacity than a particulate gel network (Urbonaite et al. 2016).

The sensory texture of gels also depends on the network. Upon ingestion, fine-stranded gels break down into large particles, when chewed and the particles do not stick to the teeth.
Particulate gels rapidly break down to small particles forming a cohesive mass that sticks to the teeth (Foegeding 2006). Hence, a range of thermal and physico-chemical conditions affect both the gelation and the formed network properties of protein gels.

4.2 Foaming and Emulsification

One of the most important aspects related to foaming and emulsification happens both before and during the foam or emulsion is prepared – that is the ability of proteins adsorbing to the interfaces.

Two different views exists on the mechanism of adsorption, with the first being the “Loop-train” model (Graham and Phillips 1979) where proteins diffuse to the interface followed by unfolding and exposure of the hydrophobic core to the interface, i.e. air-phase in foams and oil-phase in emulsions. The hydrophobic parts will orientate to the interface, while hydrophilic parts of the protein will protrude into the aqueous media (Figure 7). A more recent view on protein adsorption is the colloidal model, which build upon observations that proteins may diffuse back from the interface, only limited changes in secondary structure occur upon adsorption, and that adsorbed proteins can denature by heating thus indicating that the adsorbed proteins still exists as a globular or molten glubule forms at the interface (Wierenga and Gruppen 2010). In this model, hydrophobicity of the protein also play a pivotal role, but in this case it is the exposed hydrophobic surface patches that determine the degree and rate of adsorption (Wierenga and Gruppen 2010). By modifying proteins to have increasingly higher surface hydrophobicity was it found that high hydrophobicity results in high adsorption rates and higher amounts of adsorbed protein to the surface (Wierenga et al. 2003).

![Figure 7](image-url) Schematic representation of protein with hydrophobic core (violet) adsorption to an interface (orange) by either the “Loop-train” model where the proteins unfold at the interface and the colloidal model where the protein retains a globular structure upon adsorption. Adapted from (Wierenga and Gruppen 2010).
Adsorption rate is normally higher at the isoelectric point, suggesting that protein charge also affects adsorption. By chemically modifying ovalbumin with and increasing number of negative charges it was found that increased charge resulted in decreasing surface load and adsorption rate (Wierenga et al. 2005). Furthermore, an increase in ionic strength will also result in increased adsorption (Delahaije et al. 2014a). A fast decrease in surface tension have been associated with high foamability (Tripp et al. 1995; Marinova et al. 2009; Hammershoj et al. 1999)

Surface active molecules like proteins can lower the surface tension by adsorbing to the interface. The surface tension \( \gamma \) of a film is defined as \( \gamma = F/d \) (N/m), where \( F \) equals the surface force and \( d \) equals the length perpendicular to the force along which the force acts, i.e. surface tension is the surface force per unit length (Sears, Zemansky, & Young, 1987).

Protein adsorption and the concurrent decrease in surface tension usually follows three steps: An initial lag phase where few proteins have reached the surface, a rapid decrease in surface tension as the proteins adsorb and form a monolayer and finally a steady-state with proteins forming a thicker surface layer of packed proteins. The lag phase is dependent on protein type and concentration with low concentration resulting in a longer lag phase (Tripp et al. 1995).

A low interfacial tension is preferred since a system with high interfacial tension requires more pressure to disrupt a droplet in an emulsion or foam-bobble than a system with lower interfacial tension (\( \gamma \)) according to the Laplace pressure \( P_L = 4 \gamma / d \), where \( d \) = diameter of droplet/bubble (McClements 1999; van Koningsveld et al. 2002c).

The surface layer of proteins gives rise to an elastic film, where higher elasticity has been related to foam and emulsion stability and reduce Ostwald ripening (Blijdenstein et al. 2010; Maldonado-Valderrama et al. 2008)

A number of destabilising mechanisms, leading to collapse of foam or emulsion exists (Figure 8). For foams, this includes liquid drainage where liquid flows due to gravity resulting in a dry foam, Ostwald ripening where a high Laplace pressure on small bubbles causes diffusion of air to larger bubbles that increases in size and coalescence where the film between bobbles collapse causing formation of larger bobbles (van Koningsveld et al. 2002c). For emulsion, flocculation i.e. interaction between emulsion droplet may cause coalescence and finally creaming (Lam and Nickerson 2013). Flocculation is increased at pH near pI or at high salt
conditions, and can be counteracted by using high protein concentrations leading to high interfacial coverage or by modification of proteins leading to steric stabilisation (Delahaije et al. 2014b).

Figure 8 Illustration of foam destabilizing mechanisms starting from fresh foam. Coalescence, creaming and flocculation are also important destabilizing mechanisms for emulsions.
5 Experimental outline

This section describes the pilot plant process in use by KMC in relation to samples used for Paper I. Furthermore, overview figures of the developed purification protocol and analysis conducted with each fraction are displayed.

5.1.1 The industrial process

Figure 9 depicts a schematic representation of the pilot plant process, which is used by KMC/AKV Langholt for production of native protein. The potato fruit juice has undergone multiple processes before it enters the pilot plant, begins with washing and rasping of the potatoes, removal of potato pulp and collection of starch by rotating sieves. In the pilot plant the process starts with a deaeration step following removal of residual starch and fibres by a hydrocyclone. A ceramic filter removes additional fibre residues before the PFJ is concentrated and let to column which binds glycoalkaloids. The final steps are thermisation which lowers PPO activity and spore count, followed by spray drying which results in a powder with a typical dry matter content of 94-96% (KMC, personal communication).

![Diagram of the industrial process](image)

**Figure 9** Schematic representation of the industrial setup for production of native potato protein from PFJ. The dashed arrows indicate which constituent that is removed at a given step. Adapted from (Eriksen 2014)

5.1.2 Laboratory experiments

One objective was to produce a protein powder with a white appearance, therefore an effort was made to remove PPO early in the industrial process. Samples were collected before and after the ceramic filter (Figure 9), to assess the impact on residual fibre on ultrafiltration...
performance and separation of proteins, with a special emphasis on PPO recovery, and the results were compiled in Paper 1.

The second objective of the project was to develop and assess a purification method of potato protein. Both PFJ and spray-dried protein powder was used as start material to test the robustness of the method and to evaluate possible differences in quality parameters caused by spray drying. Furthermore the spray-dried powder was produced without (year 2014) and with (year 2015) the presence of PPO inhibitors. The developed method and assays performed on the resulting fractions are displayed in Figure 10 and Figure 11 and the results were summarised in Paper II.

The third objective was to investigate the functionality of the resulting fractions. For gelation studies both freeze- and spray-dried samples were included in order to test the impact on drying conditions, with results compiled in Paper III and V. Slight modifications were made in the production of the spray dried powder from the year 2014 to 2015 and the latter used for emulsion and foam studies since this powder was a step closer to a commercial product. The functional tests were done at different pH and ionic strength conditions to get a broader understanding of these important factors. An overview is presented in Figure 11 and the results compiled in Paper IV.
Figure 10 Overview of purification steps, by anion exchange (IEX) or hydrophobic interaction (HIC) chromatography of 2014 PFJ and 2014 spray-dried powder with indication of which assay (total phenol content, PPO activity, total glycoalkaloid) or functional test each fraction was subjected to. This figure is associated to paper II, III and V.

Figure 11 Overview of purification steps and assays performed on the resulting fractions based on 2015 spray dried powder. This figure is associated to paper II and IV.
# Overview of methods

<table>
<thead>
<tr>
<th>Aim</th>
<th>Method</th>
<th>Notes</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Determine protein concentration of solutions</td>
<td>UV/Vis absorption</td>
<td>The bicinchoninic acid assay and the Bradford assay were applied with BSA as a standard. Results obtained from Bradford assay were not reported in the papers.</td>
<td>I, II, III, IV, V</td>
</tr>
<tr>
<td>Protein separation</td>
<td>Preparative protein purification</td>
<td>Ion exchange was performed with DEAE FF at pH 8. HIC purification was performed with Butyl Sepharose High Performance at pH 8.</td>
<td>II, VI</td>
</tr>
<tr>
<td>Protein separation and concentration</td>
<td>Ultrafiltration</td>
<td>Ultrafiltration with 100 and 300 kDa filters were used for protein separation. 10 kDa filters were used for protein concentration and diafiltration.</td>
<td>I, II</td>
</tr>
<tr>
<td>Determine PPO activity in PFJ and purified fractions</td>
<td>Activity assay - UV/Vis absorption</td>
<td>A PPO activity assay was performed by mixing a protein solution with the substrate (4-methylcatechol/pyrocatechol) in a pH 6.5 buffer and the increase in absorbance at 420 nm measured.</td>
<td>I, II</td>
</tr>
<tr>
<td>Determine molecular weight of PPO</td>
<td>Activity assay - UV/Vis absorption</td>
<td>A SDS-PAGE gel was run under non-reducing conditions and active protein bands visualised by pressing the gel onto a blotting paper soaked in 4-methylcatechol.</td>
<td>I</td>
</tr>
<tr>
<td>Determine total phenol content</td>
<td>UV/Vis absorption</td>
<td>Phenols were extracted by 80% methanol and analysed by reaction with the Folin-Ciocalteu reagent at alkaline conditions or the Fast Blue BB assay (The Fast Blue BB assay was tested but results not reported in papers)</td>
<td>II</td>
</tr>
<tr>
<td>Determine dry matter in spray and freeze dried powders</td>
<td>Dry Matter</td>
<td>Approximately 2 g protein was dried by a Mettler Toledo halogen moisture analyser.</td>
<td>II, III, IV, V</td>
</tr>
<tr>
<td>Visualise protein molecular weight and pI</td>
<td>Molecular weight and pI</td>
<td>SDS-PAGE and 2-D gel electrophoresis were run using pre-cast gels under reducing conditions. Prior to 2-D gel electrophoresis samples were focused on 11 cm strips.</td>
<td>I, II, IV</td>
</tr>
<tr>
<td>Determine glycosylation pattern of patatin</td>
<td>Mass-to-charge ratio (m/z) value of ions</td>
<td>MALDI-TOF was applied in the linear mode to differentiate between molecular weight due to different glycosylation patterns</td>
<td>III</td>
</tr>
<tr>
<td>Determine the total glycoalkaloid content in protein fractions</td>
<td>HPLC</td>
<td>The two glycoalkaloids solanine and chaconine were extracted in 3% acetic acid and analysed by a HPLC method</td>
<td>II</td>
</tr>
</tbody>
</table>
Table 10 continued.

<table>
<thead>
<tr>
<th>Aim</th>
<th>Method</th>
<th>Notes</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measure colour of protein gels at different pH, and measure colour of freeze dried protein fractions</td>
<td>Colorimeter</td>
<td>Colour was measured with a Minolta Chroma meter and colours reported as Hunter L, a, b or CIE L*, a*, b* values. The L- values reflect lightness (0: black; 100: white), redness (-100: green; 100: red) and yellowness (-100: blue; 100: yellow), respectively</td>
<td>II, III</td>
</tr>
<tr>
<td>Measure emulsion droplet size</td>
<td>Particle size - laser diffraction</td>
<td>Emulsions were diluted with 1% SDS and measured by a Mastersizer</td>
<td>IV</td>
</tr>
<tr>
<td>Measure zeta-potential of protein at different pH</td>
<td>Ion mobility</td>
<td>The zeta potential was derived from measurements of the streaming potential measured by a Stabino.</td>
<td>IV</td>
</tr>
<tr>
<td>Measure emulsion activity and stability</td>
<td>Light absorbance</td>
<td>The emulsion was diluted with 0.1% SDS and an absorbance reading at 500 nm made at time 0 and after 10 minutes.</td>
<td>IV</td>
</tr>
<tr>
<td>Measure interfacial tension between air-water or oil-water</td>
<td>Force measurement</td>
<td>Interfacial and surface tension were measured with a force tensiometer based on the Wilhelmy plate method.</td>
<td>IV</td>
</tr>
<tr>
<td>Asses foam overrun and stability</td>
<td>Shaking</td>
<td>A protein solution was shaken in a 100 ml cylinder and foam height measured for two hours</td>
<td>IV</td>
</tr>
<tr>
<td>Measure water holding capacity of gels</td>
<td>Water holding capacity</td>
<td>Gel pieces were centrifuged at 10,000 × g in a centrifugal filter tube and the weight before and after centrifugation measured.</td>
<td>III</td>
</tr>
<tr>
<td>Determine stress and strain properties of gels</td>
<td>Uniaxial compression</td>
<td>Gel cylinders were cut and compressed until fracture</td>
<td>III</td>
</tr>
<tr>
<td>Determine flow properties of sheared 3% (w/w) gel</td>
<td>Rheometer flow mode</td>
<td>Shear stress was measured with a bob cup geometry by doing a shear rate sweep (up-down) ranging from 0.1-100 s⁻¹</td>
<td>V</td>
</tr>
<tr>
<td>Determine gel point and strength of gel</td>
<td>Rheometer Oscillation mode</td>
<td>Oscillatory measurements with bob cup geometry during heating and cooling</td>
<td>III &amp; V</td>
</tr>
<tr>
<td>Determine mechanical spectre of emulsion</td>
<td>Rheometer Oscillation mode</td>
<td>Oscillatory measurements with plate-plate geometry and performance of frequency sweep</td>
<td>IV</td>
</tr>
</tbody>
</table>
7 Summary of Papers I-V

Here a short introduction to the aim and results of each paper is given.

7.1 Paper I: Effect of membrane material on separation of proteins in potato fruit juice with special emphasis on polyphenol oxidase following ultrafiltration

Ultrafiltration was applied to diluted potato fruit juice. The aim was to selectively concentrate the potato proteins in the permeate, while isolating polyphenol oxidase (PPO) in the retentate. A profound difference was found in protein retention between two 300 kDa molecular weight cut-off (MWCO) ultrafiltration membranes, of either regenerated cellulose (RC) or polyethersulfone (PES). The use of the 300 kDa MWCO RC membrane resulted in a two-fold higher retentate protein content as well as total retention of all PPO activity, as compared with the PES membrane. Comparison tests with 100 and 300 kDa MWCO PES membranes indicated that concentration polarization and gel layer formation, and not MWCO definitions, were governing factors for protein retention, since proteins with MW of 10 kDa were retained in all experiments. PPO activity in potato fruit juice was measured in permeate and retentate to assess its selective retention by the applied ultrafiltration processes. Of the specific PPO activity, 94-100 % was retained by either 300 MWCO RC or 100 MWCO PES, while only 49 % specific activity was retained by the 300 MWCO PES. By in-situ blotting experiments the molecular weight of active PPO was found to be present at three different molecular weights, at positions of 40, 47 and 100 kDa, respectively, with the major activity present at 47 kDa.

7.2 Paper II: A new two-step chromatographic procedure for fractionation of potato proteins with potato fruit juice and spray dried protein as source materials

An effort to purify potato proteins with superior quality for use as food ingredients a new chromatographic procedure involving anion exchange (IEX) and hydrophobic interactions chromatography (HIC) was established. Liquid potato fruit juice (PFJ) or re-suspended spray-dried protein was separated by IEX resulting in two fractions; a protease inhibitor (PI)-rich fraction and a patatin-rich fraction. Each of these fractions were further re-chromatographed
on HIC, each resulting in two new sub-fractions. A high quality powder should have; high lightness, low polyphenol concentration and PPO activity and finally a content of glycoalkaloids below 150 ppm. The IEX PI fraction purified from spray dried powder had higher lightness values (L* = 83) than to patatin (L* = 50), whereas IEX purification from PFJ resulted in a PI fraction with decreased lightness (L* = 66) and a patatin fraction with increased lightness values (L* = 68). HIC fractionation generally led to increased lightness for patatin, but decreased lightness for the PFJ PI fractions. HIC purification significantly lowered polyphenol oxidase (PPO) activity in the fractions due to lower affinity for PPO than patatin and PI. The concentration of polyphenols was higher in the PI fraction than in patatin after IEX. HIC fractionation generally lowered polyphenol content in the patatin fractions, but increased the content in the first eluting PI HIC fraction. No correlation was found between polyphenol content of powders and lightness. Total glycoalkaloid content was below 150 ppm in three of four samples after HIC fractionation, but with α-solanine being enriched in the PI fraction, and α-chaconine in the patatin fraction. In conclusion, it was possible to obtain a PI rich protein isolate from powder with good quality attributes after both IEX and HIC, while for patatin the best quality was obtained only after the HIC, and there the colour may still be a problem.

7.3 Paper III: Gel properties of potato protein isolate and purified fractions by small-scale and large deformation rheology – impact of drying method, protein concentration, pH and ionic strength

Gel properties of potato proteins were studied under different conditions by including two drying methods for a total protein powder (freeze- and spray-drying) and a purified patatin fraction. Protein concentrations of 8 % and 15 % (w/w), pH range of 3-7.5, and 2 ionic strength levels of low (15 mM NaCl) and high (200 mM NaCl) were studied. Gels with 8 % (w/w) protein were used for oscillatory rheology measurements to assess gelation temperature, storage modulus at 85 °C and 20 °C and resistance to strain deformation. Samples prepared at conditions that promoted low solubility i.e. near pI of patatin or at pH 3 and high salt displayed structure before heating while neutral pH conditions showed gelation initiated at 40-50 °C for total protein and 55-60 °C for patatin. Gel strength expressed as the storage modulus (G′) generally increased at pH values below or above pH 4.7 for all tested
samples. Patatin showed both the lowest and the highest \( G' \) and higher elasticity of all tested samples. Large deformation textural analysis of 15 % protein gels showed both pH and ionic strength increase to resulting in firmer gels, i.e. higher axial stress. For gel elasticity, i.e. Hencky strain, the pH and ionic strength interacted. At 8 % potato protein only very weak gels were formed. The gel colour was also affected by pH, as e.g. pH 5 resulted in more white and yellowish, but less reddish gels. At pH 3.0 and ionic strength of 15 mM transparent gels could be prepared from total protein and patatin and at pH 7 I: 15 mM patatin could also produce transparent gels. The water holding capacity of gels only depended on protein concentration.

7.4 Paper IV: Foam and emulsion properties of potato protein isolate and purified fractions

Spray dried potato protein and specific isolated fractions were used for foaming and emulsification studies. Re-suspended spray dried protein was separated into a patatin and a protease inhibitor (PI) rich fraction by ion exchange chromatography (IEX), and these two fractions were purified by hydrophobic interaction chromatography into a low (HIC 1) and a high (HIC 2) hydrophobic fraction. Foam overrun for the spray dried powder and all patatin fractions were highest at pH 3, with gradually lower values at pH 5 and 7, while the PI fractions had highest overrun at pH 5 and equally lower values at pH 3 and pH 7. Relative foam stability was varying from 18-78 % of the initial foam at pH 3, with patatin forming unstable foam and the spray dried powder and PI fraction a stable foam. Lower variation in foam stability was seen at pH 5 and pH 7 with values of 67-80 %. The HIC fractions did generally perform better than the spray dried powder and IEX fractions, with patatin HIC 1 and 2 showing superior performance at pH 3 and PI HIC 2 at pH 5 and 7. Emulsions were characterized by emulsion stability and activity, emulsion droplet size and small scale dynamic rheological measurements. The PI, and especially PI HIC 1 showed bad emulsion properties with low stability, large droplet size and thinner texture. Interestingly PI HIC 2 showed much better emulsion properties on par with the spray dried powder and patatin, while showing more frequency dependent textural response. Overall the best emulsion could be made with patatin HIC 2, thus showing the importance of hydrophobicity for protein functionality.
7.5 Paper V: Appearance and textural properties of sheared potato protein isolate gels – impact of drying method, pH and ionic strength

The objective was to prepare sheared gels of potato protein concentrate and evaluate the effect of pH (3, ~4 and ~7), ionic strength (15 or 200 mM) and protein drying conditions (spray- or freeze-drying) on the final appearance and rheological characteristics. Heat-set gels (3 g total solids/100 g) at a high ionic strength (200 mM) resulted in an unhomogenous appearance with presence of clots, while low ionic strength (15 mM) gave homogenous structures. Gels prepared at pH 3 became transparent while preparation above pH 3.0 resulted in high turbidity. Heat treatment and cooling resulted in gelation for all samples except freeze-dried powder at pH 3.0. Flow curves during shear from 0.1-100 s⁻¹ were fitted by the Herschel-Bulkley model indicating shear thinning behaviour for all samples except the freeze dried sample at pH 3 which displayed a Newtonian behaviour. Oscillatory measurements after shear indicated viscus behaviour (phase angle above 45 °) for the spray dried sample at pH 3, and gelled behaviour (phase angle above 45 °) for the remaining gelled samples. Structure recovery was observed after shear in all samples except at pH 3.0. The data shows potato protein can be used as a potential ingredient in protein beverages.
8 General discussion

8.1 Protein purification

Both PFJ and re-suspended powder were used for protein purification and both start materials could be separated into relatively pure patatin and PI fractions. At an industrial scale PFJ needs to be processed immediately and fractionated during the potato harvest. If a spray dried isolate is made during the harvest, it can be further fractionation in the remaining months of the year, preferably by using some of the same equipment. Compared to PFJ, more concentrated re-suspended solutions can be made, thus decreasing the size of expensive chromatographic equipment. Re-suspended powder however needs to be concentrated and spray dried one additional time compared to PFJ which could increase cost.

Purification of either PFJ or re-suspended powder led to differences in colour of the final fractions. For PFJ the patatin fraction had an acceptable light colour and many of the dark coloured compounds could be separated by the stepwise elution on IEX. The PI fraction achieved an unacceptable dark colour, but this was mostly due to a longer processing time and due to freezing and thawing of this fraction giving PPO more time to act. If the PI fraction had been processed immediately a light colour would also be achievable. For the re-suspended powder did the PI fraction have a white hue while the patatin fraction was dark. A higher salt concentration was needed to elute bound patatin, compared to patatin in PFJ, and therefore did the coloured compounds also elute with patatin. A solution could be screening of additional chromatographic materials to assess if better separation could be found or change the stepwise gradient to two steps resulting in two patatin fractions with the first having improved colour and the second being brown.

The relative proportion of the two glycoalkaloids α-solanine and α-chaconine changed during purification by IEX and HIC. The PI fraction had a higher content of α-solanine and the patatin fraction a higher content of α-chaconine. HIC purification led to an enrichment of α-solanine in the least hydrophobic PI fraction while α-chaconine was enriched in the most hydrophobic patatin fraction, suggesting different ionic and hydrophobic properties of the two glycoalkaloid types. Both PI HIC fractions had a total glycoalkaloid content below 150 ppm, while only one patatin HIC fraction was below this threshold. The PI fractions were concentrated by ultrafiltration at pH 3 which could lead to lower glycoalkloid content.
according to an earlier published patent, so the low values might not only be a result of IEX and HIC purification but also to some degree the final concentration and diafiltration.

### 8.2 Protein determination methods

To determine the protein concentration is of fundamental importance when working with protein purification or functionality. The presence of interfering substances makes this task far from trivial. In PFJ and re-suspended powder are numerous polyphenols and oxidations products hereof present which interacts with the most common protein determination techniques. Protein concentration based on UV signal at 280 nm cannot be used due to interference with polyphenols and an increased signal if the polyphenols are bound to the proteins (Rawel et al. 2002). The most common protein determination techniques like BCA, Bradford and Lowry are all affected resulting in either under- or overestimation (Mattoo et al. 1987; Kamath and Pattabiraman 1988; Lindeboom and Wanasundara 2007). Pre-treatments like acid precipitation or binding of phenols with hydrophobic materials have been used to correct for interference and a modified Lowry method developed that also quantifies covalently bound proteins (Lindeboom and Wanasundara 2007; Winters and Minchin 2005).

Measurements were in this study conducted by the BCA method. For solutions based on spray dried powder this assay seemed to perform satisfactory and solubility data was agreeing to analysis performed at KMC (Brande, Denmark) based on the Kjeldahl method. Overestimations was however seen when PFJ and PI from PFJ was measured. The Bradford method was tested but this method let to underestimation. For future studies it is needed to perform a thorough test of different samples and protein determination methods. A modified Lowry method able to correct for bound phenolic substances was tested by a colleague but with no success.

### 8.3 Determination of total phenol content

The Folin-Ciocalteu is the most common method to estimate phenol content in foods and crops. Unfortunately can the Folin-Ciocalteu reagent react with a long list of substances e.g. proteins, vitamins, thiols, nucleotides and inorganic ions also react causing overestimation (Everette et al. 2010). A way to assess interfering substances is measurements of colour development before addition of alkali (Sanchez-Rangel et al. 2013). No colour development was appearing in the potato protein fraction tested. A surprising observation was however made with the PI proteins that dissolved and gelled in methanol and ethanol solutions used
for phenol extraction. By using more dilute suspension samples could be mixed with the Folin-Ciocalteu reagent but it is not clear to which degree this affected the assay. The Fast Blue BB assay which is claimed to only react with phenols (Medina 2011) was also tested earlier in this study on spray dried powder. The results were however not convincing, sometimes producing results with negative values and precipitates making measurement impossible. Other authors have also noted problems with this assay (Granato et al. 2016). Preliminary studies with the spectrophotometric method of (Dao and Friedman 1992) were made, and future studies of this method and the Prussian Blue assay (Granato et al. 2016) is needed to get a trustworthy result.

8.4 Functional properties – Gelation

Spray- and freeze-dried protein powder and a purified patatin fraction were tested. Unfortunately, the buffer strength of the prepared buffers appeared to be too low, and the buffer capacity of the proteins was too high, which resulted in variation in pH between samples. A number of additional studies were made to get a more complete picture of the impact of pH and ionic strength.

Spray-drying resulted in increased gel strength compared to freeze-dried samples at all tested pH and ionic strength conditions. This is a positive observation, since spray-drying is a much more economic drying process applicable for large scale than freeze-drying.

Gel strength was generally linked to solubility, with conditions promoting low solubility and yielding weak gels for all tested samples. Differences were observed between the total protein powders and the purified patatin in regards to maximum gel strength and deformability, with patatin producing gels with more elastic properties which may be a requirement in some food applications. The spray-dried powder yielded opaque gels at all conditions except pH 3.0 at an ionic strength of 15 mM. In contrast patatin could also give translucent gels at pH 7.5 at an ionic strength of 15 mM and this property could prove beneficial in some food applications.

For 3 % (w/w) sheared gels an interesting difference was observed between spray- and freeze-dried powder at pH 3, with the freeze-dried sample showing an Newtonian behavior and the spray-dried to be shear thinning.
8.5 Functional properties – Foaming and emulsification
Spray dried powder and IEX and HIC purified fractions were used for foam and emulsion studies. The spray dried powder did in general perform well, but the foam with the highest measured foam volume was a purified fraction – patatin HIC 2, the most hydrophobic patatin fraction, and the foam with highest foam stability was also a purified fraction – PI HIC 2, the most hydrophobic PI fraction. Surface tension was measured for the spray dried powder at pH 3, pH 5 and pH 7, and it was found that the rate of surface tension decrease could be correlated to foam overrun while the steady-state value could not.

For emulsions did patatin HIC 2 also perform the best of all tested fractions, while PI HIC 2 was significantly better than the other two PI fractions. Measurements of interfacial tension between oil-water showed that the HIC 2 fractions had lower interfacial tension than HIC 1 fractions, indicating the high importance of protein hydrophobicity for foaming and emulsification. The data suggests improved functional properties of the fractions thus providing a higher commercial value.

9 Conclusions
The overall aim of this study was to develop a purification strategy that resulted in high quality fractions with increased functional properties. The purification protocol developed to separate the patatin and PI fraction from each other, with both liquid PFJ and spray dried powder as start material. The colour of the final fractions was improved for the PI fraction when using spray dried powder, and the patatin fraction for liquid PFJ. HIC fractionation led to improved colour and lowering of PPO activity. Three out of four HIC fractions based on spray dried powder achieved a TGA content lower than the 150 ppm limit. These results indicate that the process is viable in producing a high quality powder, but the cost of using two chromatographic steps is a serious limitation.

It was attempted to separate the unwanted enzyme PPO selectively from the other major potato proteins by ultrafiltration. This was however not possible, but new knowledge about potato proteins and different ultrafiltration membrane materials was gained.

The functional properties of the purified fractions was compared to spray dried potato protein. New functionality was seen for patatin in high concentration protein gels, with the ability to form more elastic gels and clear gels at other pH conditions that spray dried powder.
HIC purified fractions of patatin and PI yielded superior foaming and emulsification abilities compared to spray dried powder depending on pH and ionic strength.

10 Perspectives & future studies

10.1 Protein functionality
By having a selection of fractions with different pI and ionic strength optimum can food companies’ chooser a fraction that suits their specific application best, and this will ultimately lead to incorporation of potato protein in more products.

More studies are however needed too fully understand and optimise the functionality of the different fractions. In regards to gelation are experiments with the protease inhibitor needed and as well as large deformation studies of the patatin fraction. New studies with laser or electron microscopy of gel networks could increase our knowledge and help explain differences observed between protein fractions at the tested pH and salt conditions.

The possibilities of two-step gelation i.e. heating at conditions that not favour aggregation, following change in pH or ionic strength, could also be of interest. Studies on mixtures of potato protein and common milk and egg proteins could be beneficial to pave the way for more commercial use.

For foam and emulsions new experiments concerning surface load, thin film stability and interfacial rheology at different pH and ionic strength conditions are still needed. Measurements of surface hydrophobicity of the different HIC fractions could also help to explain the obtained results and the relationship between molecular properties and functionality of the proteins.

Food applications often incorporate various polysaccharides in their formulations, therefore should future studies also map interactions with charged or neutral polysaccharides at different pH and salt conditions.

Methods that alter the secondary and tertiary structure e.g. ultrasound, limited hydrolysis or controlled denaturation by alcohol, pH or heating could also be of interest in the pursuit of enhance functionality and added value of the potato proteins.
10.2 Purification method and protein quality
The developed purification process still need polishing to yield top quality protein, with the biggest issue being the patatin fraction from re-suspended powder having a brown colour and a too high TGA content.

It is envision that the new project “proPOTATO” running in the period 2016-2021 will solve some of the remaining issues. This project is a collaboration between three departments of Aarhus University, The Department of Plant and Environmental Sciences at Copenhagen University, and the companies DuPont Nutrition Biosciences, KMC and AKV-Langholt. In this project two approaches will be explored to lower the TGA content, either by enzymatic hydrolysis or by optimization of chromatography and ultrafiltration methods. Multiple methods will be tested to inhibit PPO activity, e.g. ultrasound, limited enzymatic hydrolysis, conventional and novel inhibitors. The new protein fractions and hydrolysates will be tested for functionality and consumer perception and acceptance of potato protein will be mapped. If successful, Danish potato protein will in a foreseeable future be found in food products all over the world.
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Paper I

Effect of membrane material on separation of proteins in potato fruit juice with special emphasis on polyphenol oxidase following ultrafiltration

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Effect of Membrane Material on the Separation of Proteins and Polyphenol Oxidase in Ultrafiltration of Potato Fruit Juice

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Abstract Ultrafiltration was applied to diluted potato fruit juice, a side-stream from potato starch production. The aim of the study was to selectively concentrate the potato proteins in the permeate, while isolating polyphenol oxidase (PPO) in the retentate. A profound difference was found in protein retention between two 300-kDa molecular weight cutoff (MWCO) ultrafiltration membranes, of either regenerated cellulose (RC) or polyethersulfone (PES). The use of the 300-kDa MWCO RC membrane resulted in a twofold higher retentate protein content as well as total retention of all PPO activity, as compared with the PES membrane. Comparison tests with 100- and 300-kDa MWCO PES membranes indicated that concentration polarization and gel layer formation, and not MWCO definitions, were governing factors for protein retention, since proteins with a MW of 10 kDa were retained in all the experiments. PPO activity in potato fruit juice was measured in permeate and retentate to assess its selective retention by the applied ultrafiltration processes. Of the specific PPO activity, 94–100 % was retained by either 300 MWCO RC or 100 MWCO PES, while only 49 % specific activity was retained by the 300 MWCO PES. By in situ blotting experiments, the molecular weight of active PPO was found to be present at three different molecular weights, at positions of 40, 47, and 100 kDa, respectively, with the major activity present at 47 kDa.

Keywords Potato protein · Potato fruit juice · Polyphenol oxidase · Ultrafiltration · Membrane fouling

Introduction

Potato fruit juice (PFJ) is produced in large amounts as a side-stream during industrial production of potato starch. Typically, PFJ contains 2–5 % solids; of which, 35 % is crude protein (N-containing substances) (Knorr et al. 1977), which is in fine accordance with a total true protein content of PFJ of 1.8 % (w/w) (Zwijnenberg et al. 2002). The proteins in PFJ can roughly be divided into three groups. The first group is the patatins, representing up to 40 % of the total protein. Patatins are 39–43 kDa glycoproteins (existing as 80 kDa non-covalent dimers) with pI values of 4.45 to 5.17 and varying glycosylation patterns (Barta et al. 2012). The second group of PFJ proteins is the protease inhibitors (PI), which constitutes up to 50 % of the total protein and is divided into seven subgroups (Pouvreau et al. 2001). The molecular weight of the PI family varies widely, from 4.3 to 20.6 kDa, with pI values of 5.1–9.0 (Pouvreau et al. 2001). The third group is mainly composed of oxidative and other enzymes, like polyphenol oxidase (PPO), lipoygenase, and enzymes associated with starch synthesis (Jorgensen et al. 2011).

Potato PPO has a molecular weight of 40–69 kDa (Hunt et al. 1993; Marri et al. 2003; Eidhin et al. 2010) and a reported native multimeric structure with a mass of 340 kDa (Marri et al. 2003). The physiological role of PPO is related to plant defense, e.g., wounding of plant tissue induces increases in PPO activity (Thipyapong et al. 1995). PPO catalyzes the hydroxylation of mono-phenols to o-diphenols and oxidation...
of o-diphenols to o-quinones (Ramírez et al. 2003). Maximum activity for potato PPO is found at pH 6–6.5 at 30 °C (Eidhin et al. 2010). PPO enzyme acting on the phenolic compound, chlorogenic acid, as found in potatoes is responsible for the well-known brown color formation (Narvaez-Cuenca et al. 2013). Possible subsequent reaction products from this reaction can result in covalent and non-covalent interactions with other potato proteins, causing the final potato products, e.g., potato protein powder, to have not only a brown hue but also poorer solubility, digestibility, and content of essential amino acids (Prigent et al. 2007; Rawel et al. 2001), all leading to poorer quality. Therefore, there is a great interest in avoiding or inhibiting PPO activity during refinement of PFJ into protein products. PPO can be inhibited by heat treatment (80 °C for 15 min), low pH (pH <3.5), or by addition of antioxidants or inhibitors, like ascorbic acid or sodium metabisulfite (Eidhin et al. 2010). Acid or heat treatments are however not advisable or applicable in relation to protein extraction for food applications, due to denaturation and precipitation of some of the potato protein subfractions (Straetkvern and Schwarz 2012).

Potato proteins have been purified from PFJ by a vast number of techniques. In laboratory scale, this includes precipitation with acids (hydrochloric acid, sulfuric acid, acetic acid, citric acid), organic solvents (methanol, ethanol, 2-propanol, acetone), metal salts (FeSO₄, FeCl₃, ZnCl₂) (Barta et al. 2008), separation by ion exchange (van Koningsveld et al. 2001), affinity or hydrophobic chromatography (Racusen 1989), expanded bed adsorption, or by ultrafiltration (UF) (Straetkvern and Schwarz 2012). UF is a powerful technique for protein concentration and fractionation; problems however exist with membrane fouling due to plant fibers and proteins forming a layer on the surface or constricting the pores of the UF membranes (Eriksson and Sivik 1976; Zwijnenberg et al. 2002; Haberkamp et al. 2008). The method of purification and the conditions used will affect the functionality of the final powder, e.g., expanded bed adsorption will result in native protein with high solubility compared to that of acid-precipitated proteins, being denatured (Lokra et al. 2008).

The aim of the present study was to understand the governing factors responsible for retention of proteins from potato fruit juice during ultrafiltration. Specific focus was on testing different membrane types and molecular weight cutoff (MWCO), 100 and 300 kDa, applied to two differently processed industrial potato fruit juices. The hypothesis was that a 100-kDa filter would selectively retain the majority of the 340-kDa PPO, while permitting passage of the protease inhibitor fraction with molecular weight <21 kDa or even letting the combined fraction of patatin plus protease inhibitors with molecular weight <80 kDa pass.

### Materials and Methods

#### Materials

Two different kinds of PFJ were obtained from a local starch-processing plant (KMC, Karup, Denmark), a feed liquid containing residual fiber and cell wall material and a clarified fraction obtained after filtration on a ceramic filter. Both PFJs were from the same year of production campaign, but from different batches. The visual appearance of both PFJs was a brownish color with the feed PFJ being more turbid. The samples were kept at −18 °C until use (max 8 months). The PFJs were centrifuged at 3945g for 10 min at 4 °C in a Heraeus Multifuge 3 s-r (Kendro, Osterode, Germany) and mixed with an equal volume of 20 mM Bis-Tris buffer, pH 6.0.

#### Ultrafiltration Equipment

Three different ultrafiltration membranes (Millipore Pellicon XL 50) were tested: (1) 100-kDa MWCO Biomax(polyethersulfone (PES) (Millipore, Jaffrey, USA), (2) the same type as (1) but with MWCO of 300 kDa, and (3) 300-kDa Ultracl® regenerated cellulose (RC) (Millipore, Jaffrey, USA). The three membranes had a water cross flow at a pressure drop of 138 kPa at 50–80, 60–100, and 40–70 ml/min, respectively, as reported by the manufacturer. Two different systems were used for the experiments: a small-scale system with a 100-ml reservoir equipped with a peristaltic pump, operated to achieve a pressure drop over the membrane of 103.5 kPa and a transmembrane pressure of 134.5–138 kPa. This system was used for testing all the three membrane types and both types of the PFJ. The resulting fractions of permeate and retentate were used for protein determinations, PPO in situ blotting assay, and SDS-PAGE analysis. The second system was a commercial Millipore 29751 Labscale TFF System with 500-ml reservoir equipped with a peristaltic pump, operated to the recommended pressure drop of 138 kPa and transmembrane pressure of 138 kPa. This system was used for reconfirmation of the findings with clarified juice tested with small system. The resulting fractions were used for protein determination and SDS-PAGE.

Calculation of membrane flux \( (J) \) \( (\text{1 m}^{-2} \text{~h}^{-1}) \) was carried out by Eq. 1.

\[
J = \frac{Q_p * 60 * 10^{-3}}{\text{Area}}
\]  \( \text{(1)} \)

where \( Q_p \) is permeate flow rate \((\text{ml} \text{~min}^{-1})\) and \( \text{Area} \) refers to the cross flow area \((\text{m}^2)\) of the membrane. Transmembrane pressure (TMP) was calculated by Eq. 2.

\[
\text{TMP} = \frac{P_{\text{inlet}} + P_{\text{outlet}}}{2}
\]  \( \text{(2)} \)
Protein Content

The bicinchoninic acid assay (BCA, Thermo Scientific™ Pierce™), with bovine serum albumin (2 mg/ml) as reference protein, was used for protein determination (Smith et al. 1985). Measurements were conducted in triplicates.

SDS-PAGE Analysis

SDS-PAGE using Criterion™ TGX™ 8–16 % precast gels (Bio-Rad, Richmond, CA, USA) was performed according to (Laemmli 1970). Samples were mixed 1:1 with sample buffer (20 mM Tris, 2 % SDS, 20 % glycerol, pyronin Y) and reduced with 1/10 vol 0.2 M dithioerythritol (DTE) and boiled for 3 min; 30-μl sample of 2 mg/ml were loaded onto the gel. Samples with a higher protein concentration than 2 mg/ml were diluted prior to loading. Gels were stained with Coomassie Brilliant Blue G-250. Molecular mass was estimated by a prestained broad range molecular weight marker (Thermo Scientific™ Spectra™ Multicolor Broad Range Protein Ladders).

PPO Activity Assay

A combination of the methods by Cheng et al. (2007) and Eidhin et al. (2010) was used to analyze activity of PPO. Activity was assayed in microtiter plates with 200-μl substrate (50 mM 4-methylcatechol in 0.1 M phosphate buffer pH 6.5) and 50-μl enzyme solution. The reaction was carried out at room temperature, and an increase in absorbance at 420 nm was measured for 30 s in a Synergy 2 Microplate reader (BioTek Instruments Inc, Winooski, VT 05404, USA). One unit of activity was defined as the change in absorbance of 0.001/min/ml of enzyme solution. Specific activity was calculated by dividing the number in units with the protein concentration in milligram per milliliter. Measurements were conducted in triplicates.

PPO Blotting

In situ blotting of PPO activity was conducted essentially as described by Cheng et al. (2007). Briefly, samples were separated by SDS-PAGE as described in the above protocol, but without the addition of DTE and with lower heating (T<50 °C). A blotting paper was prepared by soaking Whatman® 40 filter paper (Whatman, Maidstone, England) in a 10 % 4-methyl catechol solution following drying for 5 min at 37 °C. SDS-PAGE analysis was performed, and the blotting paper was pressed onto the surface of the gel, without allowing any air bubbles to be present. After a few minutes, color development occurred and a picture was taken by a digital camera. The gel was finally stained with Coomassie Brilliant Blue G-250 to visualize protein bands present in the analyzed lanes.

Statistics

Origin 2015 (OriginLab, Northampton, USA) was used in the statistical analysis. One-way ANOVA was performed, and means were compared in pairs to test for significant differences at the P value of 0.05 by a Tukey test.

Results and Discussion

Ultrafiltration of Industrially Processed Potato Fruit Juices

An initial 15-min stabilization of flux was performed for all the experiments before filtration was conducted, and samples were drawn from retentate and permeate. Based on it, it can be observed that the flux is decreasing over time due to formation of a secondary gel layer and blockage of membrane pores (fouling). The flux of the feed PFJ is lower than that for the clarified PFJ when using the same 100-kDa PES membrane due to the presence of various fibers and cell wall material that quickly attach to the membrane (Fig. 1). The higher protein content of the feed PFJ (15.85 mg/ml) compared to the clarified PFJ (5.79 mg/ml) may also contribute to the observed lower flux, since an increase in the protein concentration has a limiting effect on the flux (Darnon et al. 2002). The 300-kDa RC membrane has a lower flux than the 100-kDa PES, but a similar profile over time.

Using clarified PFJ, the permeability of membranes was tested (Fig. 2). The 300-kDa RC and 100-kDa PES show almost similar profiles, while the 300-kDa PES displays a tenfold higher flux at the TMP used at the given concentration conditions of the samples at 134.5–138 kPa, indicating either a more open structure of the 300-kDa PES membrane and/or less fouling. The fluxes increased in parallel to the water cross flow reported by the manufacturer; however, the difference in flux between the 300- and the 100-kDa PES appeared larger when applying clarified potato fruit juice. The 100-kDa PES was also tested with feed PFJ, resulting in roughly 2.5-fold reduction in the observed flux, when compared to the clarified PFJ (Fig. 2).

This difference in performance between membranes types was also evident when comparing the protein content of retentate and permeate of the clarified PFJ (Table 1). The 300-kDa PES had a twofold lower protein content in the retentate compared with the 100-kDa PES and 300-kDa RC membranes, again indicating a more open structure, which will result in less membrane fouling.

The UF fractions were tested by SDS-PAGE to visualize membrane retention of proteins of different sizes. Feed PFJ
was tested with the 100- and 300-kDa PES membranes. Both membranes resulted in high retention of proteins in the retentate as seen in Table 1 and Fig. 3a. The 300-kDa membrane had a more open structure as indicated by denser bands and the presence of a 40-kDa band in the permeate (lane 4) than the 100-kDa permeate (lane 2).

For the clarified PFJ (Fig. 3b), the 100-kDa PES had a low amount of protein with a MW below 25 kDa in the permeate (lane 2) and high retention of proteins from 10–100 kDa in the retentate (lane 3). The 300-kDa PES had no apparent difference in the specific proteins between permeate (lane 4) and retentate (lane 5) as proteins from 10–100 kDa was found in both fractions. The 300-kDa RC membrane permitted passage
The 300-kDa PES membrane showed much higher retention of proteins when using feed PFJ compared to clarified PFJ. This is probably related to enhanced degree of membrane fouling due to the feed PFJ’s content of fiber and cell wall material.

**PPO Activity of Separated Fractions**

PPO activity was measured in the different fractions to analyze the retention of this specific enzyme (Table 1). For feed PFJ, 99% of the specific activity was found in retentate for the 100-kDa PES whereas 97% of the specific activity was found in the retentate of the 300-kDa membrane. The specific PPO activity was much higher in the fractions from the feed PFJ than in those from the clarified PFJ, indicating that the clarification process could lower the content of PPO. It must be noted, however, that the two PFJs were not from the same processing day, and therefore, batch to batch variation could be expected, as has been observed by the industry (KMC, personal communication). In clarified PFJ for the 300-kDa RC and 100-kDa PES membranes, PPO was highly retained, and 94–100% of the specific PPO activity was found in the retentate fraction. The 300-kDa PES membrane showed PPO activities in both the permeate and retentate with a distribution of specific activity of approximately 50/50.

The three membranes types were also tested with the Millipore 29751 Labscale TFF system to see how a larger system operated at a different pressure would affect the results. The protein content was higher in the different fractions, but the performance of the different membranes was generally observed in the small-scale experiment, i.e., the 100-kDa PES and 300-kDa RC membranes had a high retention of material in the retentate, while the 300-kDa PES membrane had a lower protein retention (Table 2) and SDS-PAGE gel (Fig. 4).

The nominal cutoff values of UF filters were found to have little influence in processing of potato fruit juice since proteins with a MW down to 10 kDa were found in the retentate in all the experiments, indicating that the secondary membrane layer made of proteins and cell wall material is a governing factor for actual retention. Differences were, however, found between the membranes used in this study with a 300-kDa PES being more permeable than a 100-kDa PES and the 300-kDa PES being more permeable than the 300-kDa RC membrane. Zwijnenberg et al. (2002) reported negligible differences when concentrating PFJ by ultrafiltration between PES, RC, and polyvinylidene fluoride membranes with MWCOs of 5 to 150 kDa. Zwijnenberg et al. (2002) suggested that the high protein content of 18 mg/ml in the PFJ could be the reason for fast build-up of material on the membrane surface and that this layer was responsible for the actual separation process.

Reduction in flux or selectivity during ultrafiltration is a well-known phenomenon in processing industries and is a complex mechanism governed by many factors. The reduction can be ascribed to reversible concentration polarization or irreversible fouling. During concentration polarization, solutes are concentrated in a boundary layer on the retentate side of the membrane, causing an increase in the osmotic pressure difference and a resulting lower driving force for transport over the membrane (Bacchin et al. 2006). Fouling can be ascribed to three different mechanisms: (i) adsorption of solutes onto the membrane surface due to specific interactions between solutes and membrane; (ii) deposit of multiple layers of material on the membrane surface resulting in a gel or cake layer on the surface; and (iii) pore blockage, where the solutes block the pores of the membrane (Bacchin et al. 2006). A difference in flux decline and fouling mechanism exists between small (<5 nm) and large (>100 nm) macromolecules (Bacchin et al. 2002). Concentration polarization is of prime importance for small macromolecules, where concentration polarization increases with increasing TMP until a critical
TMP is reached, and a gel layer is formed over the entire membrane. Concentration polarization is of less importance for large macromolecules; here, buildup of deposited material appears from the outlet to the inlet of the membrane channel when TMP is increased until a cake layer has spread over the entire membrane (Bacchin et al. 2002).

For the clarified PFJ, the observed decrease in flux may be attributed to concentration polarization, adsorption of proteins to the membrane, or pore blockage. Depending on the size of the proteins relative to the membrane pores, small proteins may enter the pores of the membrane and cause pore constriction, whereas large proteins can form a gel layer at the surface—both mechanisms result in decrease flux over the membrane (Haberkamp et al. 2008; Zhang and Ding 2015).

The lower flux of feed PFJ compared to clarified PFJ (Fig. 2) may be ascribed to additional cake formation of the larger particles present in this more crude liquid.

The observed difference between the two different 300-kDa membranes may be attributed a more open pore structure of PES membranes, but also differences in surface characteristics may contribute. Surface roughness as well as electrostatic and hydrophobic forces can affect membrane-protein interactions, e.g., adsorption of proteins to the membrane surface will be enhanced if protein and membrane have opposite charge (Haberkamp et al. 2008). The zeta potential, which gives the electric potential difference across an ionic layer around a charged colloid ion, at pH 7 has been determined for both PES and RC membranes with PES having values of $-15.1$ (Cheang and Zydney 2003), $-6.0$, and $-10.0$ (Kwon et al. 2008) and RC having values of $-2.2$ (Cheang and Zydney 2003) and $-2.9$ (Kwon et al. 2008). The lower zeta potential of the PES membranes has been associated with an electrostatic exclusion of negatively charged proteins during ultrafiltration (Cheang and Zydney 2003). In PFJ with a pH of 6, the patatins are negatively charged. Repulsion between negative patatin and the negative PES may result in a lower degree of gel formation and therefore higher permeability through the membrane. Further detailed studies at different pH and conductivity levels are, however, needed to establish the role of surface charge and protein retention with PFJ as source material.

### Visualization of PPO in PFJ by In Situ Blotting

It was not possible to selectively retain PPO in the retentate by using UF as a method to isolate this enzyme, which for reasons of solubility and browning is unwanted in potato protein products as described earlier.

Based on the results from the 300-kDa PES membrane, where PPO activity was found in both permeate and retentate, it is assumed that the active enzyme has a lower molecular weight than 340 kDa or alternatively that high shear during filtration disrupts the tertiary and quaternary structure (Schneider et al. 2007; Ashton et al. 2009). By running an SDS-PAGE gel without prior reduction of the samples, it was possible to visualize the active enzyme in the gel at distinct bands of 40 and 47 kDa and with more faint bands from 50 to 100 kDa (Fig. 5). The 40-kDa band is in agreement with data from (Eidhin et al. 2010), who purified PPO from fresh potatoes of Irish origin. Two different temperatures (20 and 50 °C) were used during sample preparation for the SDS boiling, as higher temperature may lead to increased interactions between SDS and proteins and hence different separation in the gel. It was observed, however, that the position of PPO was not affected by the applied temperatures. Heating above 50 °C may denature PPO and thus limit enzyme activity and blotting intensity (results not shown).

The presence of multiple PPO bands could be due to a.o. heterogeneity of PFJ, as it is derived from potatoes of different varieties. In the case of patatin, it has been showed to differ in pI and molecular weight between varieties (Barta et al. 2012).

### Table 2 Protein concentration of clarified PFJ after filtration in the Millipore 29751 Labscale TFF System ($n = 3$, mean ± SD)

<table>
<thead>
<tr>
<th>Membrane Type</th>
<th>Permeate</th>
<th>Retentate</th>
</tr>
</thead>
<tbody>
<tr>
<td>100-kDa PES</td>
<td>Protein (mg/ml)</td>
<td>$1.31 \pm 0.06^A$</td>
</tr>
<tr>
<td>300-kDa PES</td>
<td>Protein (mg/ml)</td>
<td>$2.9 \pm 0.03^D$</td>
</tr>
<tr>
<td>300-kDa RC</td>
<td>Protein (mg/ml)</td>
<td>$1.63 \pm 0.04^C$</td>
</tr>
</tbody>
</table>

Different letters indicate that means significantly differ at $P < 0.05$.
small proteins and formation of a gel layer of large proteins were mainly responsible for the actual separation.

In conclusion, it was not possible to selectively retain the unwanted enzyme PPO in the retentate and simultaneously let either the PI fraction with MW <21 kDa or the combination of patatin plus PI fraction with MW <80 kDa pass to the permeate. This could be due to a general fouling of the membrane or the fact that PPO was not present as a 340-kDa complex, but rather as monomers with approximate molecular weight of 40–50 kDa.

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References


A new two-step chromatographic procedure for fractionation of potato proteins with potato fruit juice and spray dried protein as source materials

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A new two-step chromatographic procedure for fractionation of potato proteins with potato fruit juice and spray dried protein as source materials

In preparation for Food and Bioprocess Technology

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Keywords: Potato protein, Patatin, Protease Inhibitor, Polyphenol Oxidase, Glycoalkaloids, Chlorogenic acid
Abstract

In an effort to purify potato proteins of superior quality for use as food ingredients a new chromatographic procedure involving anion exchange (IEX) and hydrophobic interactions chromatography (HIC) was established. Liquid potato fruit juice (PFJ) or re-suspended spray dried protein was separated by IEX resulting in two fractions; a protease inhibitor (PI)-rich fraction and a patatin-rich fraction. Each of these fractions were further re-chromatographed on HIC, each resulting in two new sub-fractions. A high quality powder should have; high lightness, low polyphenol concentration and PPO activity and finally a content of glycoalkaloids below 150 µg/g. The IEX PI fraction purified from spray dried powder had high lightness (L* = 83) compared to patatin (L* = 50), whereas IEX purification from PFJ resulted in a PI fraction with decreased lightness (L* = 66) and a patatin fraction with increased lightness (L* = 68). HIC fractionation generally led to increased lightness for patatin, but decreased lightness for the PFJ PI fractions. HIC purification significantly lowered polyphenol oxidase (PPO) activity in the fractions due to lower affinity. Concentration of polyphenols was higher in the PI fraction compared with patatin after IEX. HIC fractionation generally lowered polyphenol content in the patatin fractions, but increased the content in the first eluting PI HIC fraction. Total glycoalkaloid content was below 150 ppm in three of four samples after HIC fractionation, but with solanine being enriched in the PI fraction, and chaconine in the patatin fraction. In conclusion, it was possible to obtain a PI rich protein isolate from powder with good quality attributes after both IEX and HIC, while for patatin the best quality was obtained only after the HIC, and there the colour may still be a problem.
**Introduction**

With the world population growing rapidly and an ever increasing demand for high quality and sustainable food protein, new alternative sources like insects, algae and plants have attention. One potential source is the potato proteins which are present in potato fruit juice (PFJ), which is produced in large amounts as a side stream of potato starch manufacture. PFJ contains 2-5 % solid material, hereof 35 % crude protein (N-containing substances) (Knorr et al. 1977).

The potato proteins can be divided roughly into three overall groups; with the first group being the patatins, which make up to 40 % of the total protein. Patatin monomers have a molecular weight of 39-43 kDa, depending on N-glycosylation pattern and have pI values of 4.45 to 5.17 (Barta et al. 2012). The second group of potato proteins is the protease inhibitors (PI), which are a more diverse group, which can be divided into seven sub-groups (Pouvreau et al. 2001). The PI accounts for up to 50 % of the total protein in PFJ, and has molecular masses in a range of 4.3-20.6 kDa and with pI values of 5.1-9.0 (Pouvreau et al. 2001). The third group of potato proteins is composed of oxidative enzymes, including polyphenol oxidase (PPO), lipoxygenase and enzymes functioning in starch synthesis (Jorgensen et al. 2011). Potato PPO is, in food contexts, an unwanted enzyme catalyzing the hydroxylation of mono-phenols to o-diphenols and further oxidation of formed o-diphenols into o-quinones (Ramírez et al. 2003). The presence in potatoes of PPO combined with presence of the major phenolic compound, chlorogenic acid, is responsible for formation of undesired brown color (Narvaez-Cuenca et al. 2013). Possible covalent- and non-covalent interactions between phenolic compounds and potato proteins may cause the final potato protein powder to have a brown appearance, but also decreased solubility and digestibility (Prigent et al. 2007; Rawel et al. 2001), thus resulting in poor quality. It is therefore of interest to inhibit PPO activity in the PFJ used for protein isolation or separate the quality decreasing enzyme from the other proteins during the processing steps.

Heat treatment (80 °C for 15 min), low pH (pH < 3.5), addition of ascorbic acid or sodium metabisulfite can each inhibit PPO (Eidhin et al. 2010). Low pH and heat treatments of PFJ can, however, also result in denaturation and precipitation resulting in protein not suitable for human consumption (Straetkvern and Schwarz 2012). Additionally, in relation to its use as a source of food protein, potatoes contain the unwanted metabolites including the steroidal glycoalkaloids, α-solanine and α-chaconine. These compounds confer a
bitter taste and possible toxicological reactions, like gastrointestinal disturbance, neurological disorders or even death if consumed in large amounts (Alt et al. 2005). The EU-commission has therefor set a limit of 150 ppm total glycoalkaloids (TGA) in the final protein powder (Byrne, 2002.)

Concentration and separation of the proteins in PFJ have been done in a number of ways. This includes precipitation with acids (citric acid, acetic acid, hydrochloric acid, sulfuric acid), organic solvents (methanol, ethanol, 2-propanol, acetone) and metal salts (FeSO₄, FeCl₃, ZnCl₂) (Barta et al. 2008). Separation techniques like ion exchange (van Koningsveld et al. 2001) or hydrophobic interaction chromatography (Racusen 1989), expanded bed adsorption or ultrafiltration have been employed (Straetkvern and Schwarz 2012). Ultrafiltration with different membrane materials and molecular cut-off values cannot separate the unwanted PPO from the other potato proteins (Schmidt et al. 2016). Ion exchange and expanded bed adsorption result in one or two fractions, i.e. a total protein isolate or a separated patatin and PI fraction. Further purification of the obtained patatin and the PI protein isolates have been reported only scarcely in the literature (Pots et al. 1999; Pouvreau et al. 2001). The aim of the present study was therefore to explore the potential benefits of combining ion exchange (IEX) and hydrophobic interaction chromatography (HIC) on protein fractionation of major potato proteins and characterize quality parameters like colour, glycoalkaloids and phenol content as well as PPO activity in the final protein fractions. The aim was further to test the robustness of the method by using both liquid potato fruit juice or suspensions of spray dried potato protein as source of starting material.
Materials and Methods

Materials

Liquid potato fruit juice (stored at -18 °C) and spray dried potato protein isolate powders from the harvest 2014 were provided by KMC (Brande, Denmark) and used for the studies. Furthermore, a powder from 2015 was also analysed, this new powder was made with the inclusion of sodium bisulphite to inhibit PPO activity during manufacturing at the plant.

Chromatographic protein purification

The purifications were carried out using liquid potato fruit juice or re-solubilised spray dried protein powder. A two-step procedure was developed consisting of first an anion exchange column followed by hydrophobic interaction chromatography (HIC). Starting with potato fruit juice on IEX, the liquid was adjusted to pH 8 with 6 M NaOH and centrifuged (45 min, 5000 x g, 4 °C) to remove insoluble material. One and a half liter was loaded with a flow rate of 17 ml/min onto a XK 50 column (GE Healthcare, Uppsala, Sweden) with a 980 ml packed bed volume of DEAE Sepharose Fast Flow medium (GE Healthcare, Uppsala, Sweden) equilibrated with a 25 mM Tris-HCL buffer, pH 8 (buffer A). The unbound fraction (PI-rich) was washed out with 1.2 column volumes (CV) of buffer A. Elution of the bound fraction (patatin rich) was conducted with 25 mM Tris-HCL, pH 8.0, with 1 M NaCl (buffer B) in two steps, with 28 (0.9 CV) and 100 (1.5 CV) % buffer B. The chromatographic system was an ÄKTA purifier 100 (GE Healthcare, Uppsala, Sweden).

For the powder on IEX, suspensions of 30 g/L (w/v) in buffer A adjusted to a conductivity of 10 mS/cm by addition of NaCl were stirred for one hour. Suspensions were centrifuged (25 min, 25000 x g, 4 °C) and filtered to remove undissolved protein. The column was equilibrated with 9 % buffer B and 1150 ml loaded following elution with 0.9 CV 35 % buffer B and 1.5 CV 100 % buffer B. Both the bound (patatin rich) and unbound (PI rich) fractions were loaded at a flow rate of 7 ml/min on a XK 26 column with a 175 ml packed bed volume of HIC Butyl Sepharose High Performance beads (GE Healthcare, Uppsala, Sweden). For liquid potato fruit juice on HIC, the patatin rich fraction was adjusted to a conductivity of 110 mS/cm with NaCl and 200 ml loaded onto the column previously equilibrated with 25 mM Tris-HCL buffer adjusted to 110
mS/cm (buffer B). The column wash was washed with 1 CV buffer B and two fractions collected by elution with demineralized water with the following gradient: 1 CV 21% B and 2 CV 0% buffer B. The PI fraction was adjusted to 145 mS/cm, centrifuged (25 min, 25000 x g, 4 °C) and 550 ml loaded and eluted with the following gradient: 1 CV 2% B and 2 CV 0% B.

The patatin fraction from spray dried powder for HIC was adjusted to 135 mS/cm and eluted with the following 2-step gradient: 1 CV 19% B and 2 CV 0% buffer B. The PI fraction was adjusted to 145 mS/cm and processed as described above, but with the following 2-step gradient: 1 CV 6% B and 2 CV 0% buffer B.

**Ultra- and diafiltration**

Ultrafiltration and diafiltration was performed with a PALL UltraLab™ system equipped with a 10 kDa molecular weight cut off Minimate membrane (PALL, New York, USA). All patatin fractions were concentrated at pH 8 and diafiltered with demineralized water to a final conductivity of 1.2-1.7 mS/cm. All PI fractions were adjusted to pH 3 with 6 M HCl following concentration and diafiltration to a conductivity of 1.2-1.7 mS/cm. After diafiltration, the samples were freeze dried and stored at -18 °C.

**pH and conductivity measurements**

pH of the solutions was analyzed by a PHM 92 pH-meter (Radiometer, Copenhagen, Denmark) and conductivity measured at 25 °C by a CDM 210 Conductivity Meter (Radiometer, Copenhagen, Denmark).

**Protein concentration determination**

Protein concentration was determined by the bicinchoninic acid assay (BCA, Thermo Scientific™ Pierce™), with bovine serum albumin (2 mg/mL) as reference protein for protein determination (Smith et al. 1985). Measurements were conducted in triplicates.

**Determination of glycoalkaloids**

Total glycoalkaloids (TGA) constituting solanine and chaconine were determined by the HPLC method as described (Hellenas and Branzell 1997) with extraction of the protein sample in 3% acetic acid followed by solid phase extraction.
**Polyphenol oxidase activity assay**

A combination of the methods by (Cheng et al. 2007) and (Eidhin et al. 2010) was used to analyse the activity of PPO. Activity was assayed in microtiter plates with 200 µl substrate (50 mM pyrocatechol in 0.1 M phosphate buffer, pH 6.5) and 50 µl enzyme solution. The reaction was carried out at room temperature, and an increase in absorbance at 420 nm was measured for 30 seconds in a Synergy 2 Microplate reader (BioTek Instruments Inc, Winooski, VERMONT 05404 USA). One unit of activity was defined as the change in absorbance of 0.001 per min per mL of enzyme solution. Specific activity was calculated by dividing the number in units with the protein concentration in mg/mL. Measurements were conducted in triplicates.

**Total Phenolic content by Folin-Ciocalteu assay**

Total phenol determination was prepared as described by (Sanchez-Rangel et al. 2013) with some modifications. 100-500 mg protein powder was suspended in 3-9 ml 80 % methanol and mixed by Ultra-Turrax (Polytron PT 2100) for 1 min followed by 2 hour shaking. The sample was centrifuged 10 min at 20800 × g in a table top centrifuge and the supernatant used for the assay. The assay was performed in 96-well microplates. 15 µL extract was mixed with 235 µL H₂O and 20 µL 1 N Folin-Ciocalteu reagent. 30 µL 0.5 M Na₂CO₃ was added and incubation was done for 2 h at room temperature in darkness. Absorbance was measured at 765 nm and compared to a standard curve of gallic acid. Results were expressed as mg gallic acid equivalence per g protein powder (mg GAE g⁻¹).

**Colour measurement of powder**

Colour of freeze dried powder, ground by hand, was measured with a Minolta Chroma Meter CR-400 (Konica Minolta, Osaka, Japan) using the CIE (Commision Internationale de L’Enclairage) Lab scale calibrated against standardized daylight (D65). The L*, a* and b* values correspond to lightness (0: black; 100: white), redness (-100: green; 100: red) and yellowness (-100: blue; 100: yellow). Measurements were conducted in quadroduplicates. A photo of the different freeze dried fractions was taken with a digital camera.
One dimensional gel electrophoresis

One dimensional SDS-polyacrylamide gel electrophoresis (1-DGE) using Criterion™ TGX™ 8-16 % precast gels (Bio-Rad, Richmond, CA, USA) was carried out as described (Laemmli 1970). Samples were mixed 1:1 with sample buffer (20 mM Tris, 2 % SDS, 20 % glycerol, pyronin Y), reduced with 1/10 vol 0.2 M dithioerythritol (DTE) and boiled for 3 min. Thirty µL of sample was loaded in each well. Gels were stained with Coomassie Brilliant blue G-250. Molecular mass was estimated by a prestained broad range molecular weight marker (Thermo Scientific™ Spectra™ Multicolor Broad Range Protein Ladders).

Two dimensional gel electrophoresis

Two dimensional SDS-PAGE (2-DGE) was conducted essentially as described previously (Jensen et al. 2012) with focusing of 100 µg protein on either pH 4-7 or pH 5-8 immobilized gradient strips and running on 8-16 % polyacrylamide gels under reducing conditions. The gels were stained by Coomassie Brilliant blue G-250.

MALDI-TOF mass spectrometry

Masses of HIC purified patatin fractions were determined by MALDI-TOF mass spectrometry (MS) by recording mass spectra of positively charged ions in linear mode as described previously (Barta et al. 2012). Protein samples were mixed with DHB matrix solution (90% 2,5-dihydroxybenzoic acid and 10% 2-hydroxy-5-methoxybenzoic acid; 40 mg/mL in 20% acetonitrile and 1% trifluoroacetic acid) and droplets applied to a MALDI target plate.
Results & Discussion

Schematic figure of separation flow

Fig. 1 Schematic representation of purification flow and the various analysis conducted on the separated protein fractions.

Fig. 1 depicts the fractions obtained after IEX and HIC purification, respectively, with PFJ or spray dried powder as source materials as well as experiments performed on the resulting fractions.

Elution profiles of IEX and HIC separations

Fig. 2a and b show IEX purification of PFJ and re-suspended powder, respectively. In both chromatograms the first major peak (flow through/FT) corresponds to unbound protein, rich in protease inhibitor (PI). The second peak represents bound material rich in patatin, while the third peak is rich in phenolic compounds, especially for the PFJ sample, as indicated by high absorbance values at 320 nm and 400 nm (Narvaez-Cuenca et al. 2013). As indicated in the 1-DGE Fig. 3a and b, lane 4 the third fraction is low in protein, which is in contrast to the relatively high signals at 280 nm in the chromatogram.

Table 1 shows integrated the peak areas under the curve at the different wavelengths, and it is seen that IEX purification of PFJ is able to separate the majority of phenolic compounds i.e. chlorogenic acid (320 nm) and especially the oxidized phenolics (400 nm) from the main protein peaks.
Fig. 2c and e display the PI and patatin fractions from PFJ, further purified by HIC. Both chromatograms have significant signals at both 280, 320 and 400 nm in the flow through, but as seen in the 1-DGE picture Fig. 3a, b, lane 4 these fractions are quite low in protein, though presents the majority of both phenolics and oxidized phenolics (Table 1). The large signal at 280 nm may be due to phenolics bound to proteins, since derivatization of protein with phenolic acids increases absorption compared to pure protein (Rawel et al. 2002). The HIC chromatograms for powder (Fig. 2d, f) show significantly lower signals in the flow through, but relatively higher values at 320 and 400 nm, co-eluting with the bound proteins (Table 1).
Fig. 3 1-DGE gels of purified fractions based on a PFJ or b re-solubilised powder. Dilution factors or concentration of samples are in brackets. a Lane 1 PFJ (10X); Lane 2 IEX FT (4X); Lane 3 IEX bound proteins (10X); Lane 4 high salt (4500 – 5000 mL in Fig 2a) (0X); Lane 5 HIC FT patatin (0X); Lane 6 HIC peak 1 patatin (10X); Lane 7 HIC peak 2 patatin (40X); Lane 8 HIC FT PI (0X); Lane 9 HIC peak 1 PI (10X); Lane 10 HIC peak 2 PI (10X). b Lane 1 powder (~0.5 mg/mL); Lane 2 IEX FT (4X); Lane 3 IEX bound proteins (16X); Lane 4 high salt (3800-4000 mL in Fig. 2b) (0X); Lane 5 HIC FT patatin (0X); Lane 6 HIC peak 1 patatin (16X); Lane 7 HIC peak 2 patatin (50X); Lane 8 HIC FT PI (0X); Lane 9 HIC peak 1 PI (10X); Lane 10 HIC peak 2 PI (~0.5 mg/mL).

Table 1 Relative values (% of total area) of integrated peak areas from under the curves of flow through and the two eluting peaks of IEX and HIC chromatograms for PFJ and re-suspended powder.

<table>
<thead>
<tr>
<th>Chromatogram</th>
<th>Wavelength (nm)</th>
<th>Flow through</th>
<th>Peak 1</th>
<th>Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEX purification of PFJ</td>
<td>320/400</td>
<td>32/11</td>
<td>20/12</td>
<td>48/78*</td>
</tr>
<tr>
<td>HIC purification of PFJ PI</td>
<td>320/400</td>
<td>64/52</td>
<td>16/18</td>
<td>20/30</td>
</tr>
<tr>
<td>HIC purification of patatin PI</td>
<td>320/400</td>
<td>77/70</td>
<td>10/13</td>
<td>13/17</td>
</tr>
<tr>
<td>IEX purification of powder</td>
<td>320/400</td>
<td>27/24</td>
<td>64/68</td>
<td>8/8</td>
</tr>
<tr>
<td>HIC purification of powder PI</td>
<td>320/400</td>
<td>34/9</td>
<td>32/34</td>
<td>34/57</td>
</tr>
<tr>
<td>HIC purification of powder patatin</td>
<td>320/400</td>
<td>26/23</td>
<td>30/30</td>
<td>44/47</td>
</tr>
</tbody>
</table>

*The signal at 320/400 nm was above the detection limit.

The recovery during the two purification steps is summarized in Table 2. The majority of protein after IEX purification of powder is found in the patatin fraction, while the PI fraction is largest after purification of PFJ. For HIC purification of patatin, the majority of protein is found in the second eluting fraction (patatin HIC 2), while the majority of protein for HIC purification of the PI fraction is found in the first eluting peak (PI HIC 1). The relatively low recovery during HIC purification of PFJ protease inhibitors is believed to be caused by overestimation of protein in the injected PI fraction, due to its high concentrations of phenolic substances which can interact with common protein determination methods like BCA, Bradford or Lowry (Lindeboom and Wanasundara 2007; Mattoo et al. 1987; Kamath and Pattabiraman 1988). Based on the 1-DGE gel (Fig. 3a,b) recovery of all fractions should be high with minor losses in the high salt fraction (Lane 4) and minor loss in flow through for HIC purification. In conclusion, the main potato proteins, either as re-
solubilized powder or as PFJ, can be separated and purified by a new combination of IEX and HIC yielding equivalent isolated protein fractions, though use of PFJ as start material leads to better separation of phenolic compounds from protein, as based on integrated peak areas at 320 nm and 400 nm compared with the results obtained for re-solubilised spray dried potato protein powder.

Table 2 Recovery and protein content in the different fractions obtained during purification of 2014 powder or 2014 PFJ as calculated based on protein concentration in fractions as determined by the BCA assay as well as the volume in each fraction. Peak 1 in IEX corresponds to the flow through fraction (PI) and peak 2 is the bound protein (patatin). For HIC purification peak 1 corresponds to the bound protein in fractions HIC 1 and peak 2 to protein in HIC fraction 2.

<table>
<thead>
<tr>
<th>Method and start material</th>
<th>Injected (g)</th>
<th>Peak 1 (g)</th>
<th>Peak 2 (g)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IEX purification of PFJ</td>
<td>21.99</td>
<td>10.76</td>
<td>5.36</td>
<td>73.31</td>
</tr>
<tr>
<td>HIC purification of PFJ PI</td>
<td>3.09</td>
<td>0.65</td>
<td>0.33</td>
<td>31.72</td>
</tr>
<tr>
<td>HIC purification of patatin PI</td>
<td>1.64</td>
<td>0.34</td>
<td>0.81</td>
<td>70.12</td>
</tr>
<tr>
<td>IEX purification of powder</td>
<td>27.24</td>
<td>10.19</td>
<td>13.41</td>
<td>86.64</td>
</tr>
<tr>
<td>HIC purification of powder PI</td>
<td>2.66</td>
<td>1.11</td>
<td>0.58</td>
<td>63.53</td>
</tr>
<tr>
<td>HIC purification of powder patatin</td>
<td>2.89</td>
<td>0.55</td>
<td>1.41</td>
<td>67.82</td>
</tr>
</tbody>
</table>

2-DGE of HIC purified fractions

The patatin and PI fractions purified by HIC were separated by 2-DGE depicted in Fig. 4a-d. The patatin HIC 1 and 2 fraction had different patatin and PI profiles (marked by arrows). The PI HIC 1 fraction (Fig. 4c) had a prominent spot at pH 6, and multiple spots around 10 kDa which was absent in the PI HIC 2 gel. In contrast, PI HIC 2 presented several dense spots at pH 7. A large proportion of the proteins in the HIC 1 fraction had pI values above pH 8, in contrast to pI HIC 2.
Analysis of molecular masses of patatins by MALDI-TOF MS

HIC purification resulted in two patatin fractions with the second eluting fraction being enriched in a high molecular weight isoform of patatin (Fig. 3a and b lane 7). The four patatin HIC fractions were analysed by MALDI-TOF MS in linear mode. The obtained masses are reported in Table 3, and an example of a representative mass spectrum is shown in Fig 5. The masses of the different isoforms correspond to one, two or three glycosylations with previously reported masses of 40513-40666, 41702-41884 and 42884-42975 Da, respectively (Barta et al. 2012). The isoform containing three glycosylations have been reported not being present in all potato varieties, but it is found in relatively high proportions in the cultivar “Kuras” (Barta et al. 2012), which is commonly grown in Denmark. The mass spectra (Fig. 5) indicate additional peaks with m/z values of 4700-4900. These peaks may be associated with covalently bound phenolics since binding of one chlorogenic acid molecule inducing a mass shift of 353.3 (Ali et al. 2013).
Table 3 MALDI TOF MS results of patatin isoforms present in HIC fractions from PFJ and 2014 powder.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak 1 (Da)</th>
<th>Peak 2 (Da)</th>
<th>Peak 3 (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFJ patatin HIC 1</td>
<td>40596</td>
<td>41812</td>
<td>42948</td>
</tr>
<tr>
<td>PFJ patatin HIC 2</td>
<td>40594</td>
<td>No peak</td>
<td>42952</td>
</tr>
<tr>
<td>Powder Patatin HIC 1</td>
<td>40600</td>
<td>41818</td>
<td>42956</td>
</tr>
<tr>
<td>Powder Patatin HIC 2</td>
<td>40595</td>
<td>41833</td>
<td>42950</td>
</tr>
</tbody>
</table>

Fig. 5 MALDI-TOF mass spectra from 2014 powder fractions, a patatin HIC 1, b patatin HIC 2.

TGA content in the separated fractions

Table 4 Content of TGA and individual glycoalkaloids solanine and chaconine present in the different protein fractions from 2014 spray dried powder.

<table>
<thead>
<tr>
<th>Sample</th>
<th>TGA (µg/g)</th>
<th>Solanine (µg/g)</th>
<th>Chaconine (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spray dried powder</td>
<td>91</td>
<td>48,7 ± 2,3</td>
<td>42,3 ± 3,3</td>
</tr>
<tr>
<td>PI IEX</td>
<td>39,1</td>
<td>32,8 ± 3,3</td>
<td>6,3 ± 0,6</td>
</tr>
<tr>
<td>Patatin IEX</td>
<td>170,3</td>
<td>44,1 ± 1,7</td>
<td>126,2 ± 2,0</td>
</tr>
<tr>
<td>PI HIC 1</td>
<td>65,8</td>
<td>61,3 ± 0,3</td>
<td>4,5 ± 1,25</td>
</tr>
<tr>
<td>PI HIC 2</td>
<td>13,6</td>
<td>3,7 ± 0,6</td>
<td>9,9 ± 1,3</td>
</tr>
<tr>
<td>Patatin HIC 1</td>
<td>60,4</td>
<td>29,3 ± 0,4</td>
<td>31,1 ± 1,4</td>
</tr>
<tr>
<td>Patatin HIC 2</td>
<td>217,9</td>
<td>53,1 ± 0,9</td>
<td>164,8 ± 0,1</td>
</tr>
</tbody>
</table>

\(n = 2-4\), means ± SD). Different letters indicate significantly different means \((P<0.05)\)

The content of solanine, chaconine and the total sum of the two glycoalkaloids (TGA) are summarized in Table 4. The PI fractions had a 2.3 fold lower total TGA content compared with spray dried powder, whereas the patatin fraction had a 1.9 fold increase. When looking at individual glycoalkaloids, solanine was associated with the PI fraction, while chaconine was associated with the patatin fraction. Further purification by HIC led to an enrichment of TGA in PI HIC 1 (low hydrophobicity) compared to PI HIC 2 (high
hydrophobicity). For patatin HIC lead to an enrichment of TGA in the patatin HIC 2 fraction. The difference between solanine and chaconice contents upon purification suggests varying interaction mechanisms between TGA-column and/or TGA-protein. Other researchers have also reported TGA content in potato protein after purification: In a study comparing ultrafiltration or expanded-bed chromatography the resulting powders displayed with 213 ± 100 and 286 ± 95 µg/g TGA, respectively (Straetkvern and Schwarz 2012). Expanded-bed can, however, result in a low TGA content, below 50 µg/g (Lokra et al. 2008). Purification of PFJ by cationic clay minerals has also proven favourable results with reduction in α-solanine by > 90 % of initial values (Ralla et al. 2012).

**Total phenolic content in the separated fractions**

Total phenol content was determined by the Folin-Ciocalteu assay in the purified fractions based on PFJ and 2014 spray dried powder (Table 5). Similar trends were observed for both powder and PFJ, with the highest content in the PI fraction and lowest in the patatin fraction following IEX purification. Based on Fig. 2a, b and the integrated values under the curve in Table 1, it would have been expected that the patatin fraction purified from powder would have higher values than the PI fraction. Further purification by HIC of the PI fraction led to concentration of phenolics in PI HIC 1, compared to HIC 2. This also contradicts the data in Table 1 and it would have been expected that HIC purification of PFJ PI had a lowering effect since many phenolic compounds eluted in the flow through fraction. The patatin HIC fractions all had lower concentrations of phenolics than the IEX patatin fraction. It was observed that upon mixing 80 % methanol (or 80 % ethanol) with the PI fractions of 2014 powder, clear gels would form within 30 minutes when 500 mg protein was mixed with 3 mL solvent. This may explain the high values for the PI fractions since proteins also reacts with the Folin-Ciocalteu reagent (Lindeboom and Wanasundara 2007). Other compounds e.g. ascorbic acid and reducing sugar may also react with the Folin-Ciocalteu reagent, inducing blue colour formation before addition of alkali (Sanchez-Rangel et al. 2013), but in our case no blue colour was observed for any of the tested samples.
Table 5 Determination of total phenolic content in the different obtained fractions expressed as mg gallic acid equivalence per gram.

<table>
<thead>
<tr>
<th></th>
<th>2014 PFJ (mg GAE g⁻¹)</th>
<th>2014 powder (mg GAE g⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spray dried powder</td>
<td>nd</td>
<td>0.15 ± 0.01</td>
</tr>
<tr>
<td>PI IEX</td>
<td>3.12 ± 0.28</td>
<td>6.37 ± 0.52</td>
</tr>
<tr>
<td>Patatin IEX</td>
<td>0.79 ± 0.01</td>
<td>0.38 ± 0.01</td>
</tr>
<tr>
<td>PI HIC 1</td>
<td>7.51 ± 0.39</td>
<td>9.77 ± 1.05</td>
</tr>
<tr>
<td>PI HIC 2</td>
<td>2.54 ± 0.25</td>
<td>6.45 ± 0.74</td>
</tr>
<tr>
<td>Patatin HIC 1</td>
<td>0.13 ± 0.01</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>Patatin HIC 2</td>
<td>(below detection limit)</td>
<td>(below detection limit)</td>
</tr>
</tbody>
</table>

(n = 2-3, means ± SD). Different letters indicate significantly different means (P<0.05).

**PPO activity in the purified fractions**

PPO activity was determined directly in the liquid fractions after IEX and HIC purification of 2014 re-suspended powder and PFJ (Table 6) and for the freeze dried fractions after ultrafiltration/diafiltration of 2014 and 2015 powder and PFJ (Table 7). Following IEX purification of re-suspended 2014 powder or PFJ, PPO was predominantly found in the PI fraction with a higher specific activity in this fraction compared with the patatin fraction. High specific activity was found in the HIC flow through fraction of both PI and patatin, indicating that PPO had a lower binding affinity to the HIC column than both patatin and PI. When comparing specific activity of the “liquid” fractions with the freeze dried fractions in a significant decrease in specific activity is seen for the PI fractions. This drop is attributed to pH induced deactivation of PPO, since the PI fractions are adjusted to pH 3 prior to concentration/diafiltration. In conclusion, HIC purification resulted in significantly lower PPO activity in the freeze dried fractions of all tested samples, due to an apparent selective binding of patatin and PI to the HIC column compared to PPO.
Table 6 PPO activity measured on the liquid fractions from IEX and HIC purification before pH adjustment and freeze drying.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity U (ml/min)</td>
<td>Specific activity (U/mg)</td>
<td>Activity U (ml/min)</td>
<td>Specific activity (U/mg)</td>
</tr>
<tr>
<td>PFJ</td>
<td>10504 ± 11</td>
<td>1576 ± 2</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>PI IEX</td>
<td>4648 ± 108</td>
<td>1656 ± 38</td>
<td>2536 ± 23</td>
<td>524 ± 5</td>
</tr>
<tr>
<td>Patatin IEX</td>
<td>8416 ± 79</td>
<td>1028 ± 10</td>
<td>880 ± 11</td>
<td>53 ± 1</td>
</tr>
<tr>
<td>PI HIC flow through</td>
<td>2816 ± 79</td>
<td>1252 ± 32</td>
<td>424 ± 11</td>
<td>1070 ± 29</td>
</tr>
<tr>
<td>PI HIC 1</td>
<td>2768 ± 30</td>
<td>551 ± 6</td>
<td>376 ± 45</td>
<td>49 ± 5</td>
</tr>
<tr>
<td>PI HIC 2</td>
<td>2312 ± 108</td>
<td>416 ± 19</td>
<td>1608 ± 52</td>
<td>203 ± 2</td>
</tr>
<tr>
<td>Patatin HIC flow through</td>
<td>2384 ± 23</td>
<td>4567 ± 43</td>
<td>240 ± 0</td>
<td>594 ± 0</td>
</tr>
<tr>
<td>Patatin HIC 1</td>
<td>948 ± 83</td>
<td>365 ± 32</td>
<td>256 ± 45</td>
<td>60 ± 11</td>
</tr>
<tr>
<td>Patatin HIC 2</td>
<td>2160 ± 39</td>
<td>201 ± 4</td>
<td>296 ± 73</td>
<td>22 ± 6</td>
</tr>
</tbody>
</table>

(n = 4, means ± SD). Different letters indicate that means significantly differ at P<0.05.

Table 7 PPO activity of freeze dried fractions purified from spray dried powder from 2014 and 2015, and 2014 PFJ.

<table>
<thead>
<tr>
<th></th>
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<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity U (ml/min)</td>
<td>Specific activity (U/mg)</td>
<td>Activity U (ml/min)</td>
<td>Specific activity (U/mg)</td>
<td>Activity U (ml/min)</td>
<td>Specific activity (U/mg)</td>
</tr>
<tr>
<td>Spray dried powder</td>
<td>nd</td>
<td>nd</td>
<td>2880 ± 98</td>
<td>675 ± 23</td>
<td>2112 ± 0</td>
<td>168 ± 0</td>
</tr>
<tr>
<td>PI IEX</td>
<td>4336 ± 261</td>
<td>507 ± 31</td>
<td>632 ± 23</td>
<td>58 ± 2</td>
<td>824 ± 23</td>
<td>79 ± 2</td>
</tr>
<tr>
<td>Patatin IEX</td>
<td>10088 ± 49</td>
<td>835 ± 4</td>
<td>1464 ± 34</td>
<td>102 ± 2</td>
<td>584 ± 23</td>
<td>41 ± 2</td>
</tr>
<tr>
<td>PI HIC 1</td>
<td>856 ± 49</td>
<td>92 ± 5</td>
<td>160 ± 30</td>
<td>13 ± 3</td>
<td>176 ± 12</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>PI HIC 2</td>
<td>336 ± 38</td>
<td>36 ± 4</td>
<td>256 ± 23</td>
<td>19 ± 2</td>
<td>176 ± 12</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>Patatin HIC 1</td>
<td>2416 ± 23</td>
<td>208 ± 2</td>
<td>504 ± 34</td>
<td>35 ± 2</td>
<td>184 ± 60</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>Patatin HIC 2</td>
<td>2144 ± 41</td>
<td>159 ± 3</td>
<td>248 ± 30</td>
<td>17 ± 2</td>
<td>216 ± 34</td>
<td>15 ± 3</td>
</tr>
</tbody>
</table>

(n = 3, means ± SD). Different letters indicate significantly different means (P<0.05).

Colour analysis of freeze dried samples

A picture of the freeze dried fractions is displayed in Fig. 6 and the colour expressed as L*, a* and b* is shown in Fig. 7 a,b and c. The purified fractions with powder as starting material resulted in PI fractions with high values of lightness, low positive a*-values and b* values of ~11, indicating a yellow colour. HIC fractionation did not lead to further changes in L* for the 2015 powder, though a slight decrease in L* for PI HIC 2 for 2014 powder. The a* values changed to a negative (more green) for both 2015 PI HIC fractions and a higher positive a* (more red) for the 2014 PI HIC fractions, while b* decreased (more blue) and increased (more yellow) for 2015 and 2014 PI HIC fractions, respectively. The PI IEX fraction based on PFJ had a significantly lower L* and higher a*-value than fractions based on powder. Lightness decreased and a* increased of the PFJ PI HIC fractions as a possible outcome of high PPO activity during purification.
The patatin fractions based on 2014 and 2015 powder had a lower lightness and a brown hue (Fig. 6). HIC fractionation resulted in higher lightness for HIC 1 for both powders. The patatin fraction based on PFJ had a significantly higher lightness and yellowness than the powder patatin fractions, and lightness was further increased upon HIC fractionation. HIC fractionation also resulted in a significant drop in the $a^*$-values for the PFJ patatin. (Straetkvern and Schwarz 2012) found a negative correlation ($R^2 = 0.871$) between lightness ($L^*$) and the content of phenolics of freeze dried potato protein, but no correlation was found in this study when comparing the values in Table 5 and lightness (Fig. 7a).

Preliminary studies by size-exclusion chromatography on a Sephacryl S-200 HR column of the patatin fraction from spray dried powder showed high signals at 320 nm and 400 nm eluting prior to the patatins, indicating that the coloured complexes have a larger size than the 80 kDa patatin dimers, and that colour can be removed from this fraction. In conclusion, fractionation of powder by IEX resulted in a light PI fraction and a dark patatin fraction, while IEX fractionation of PFJ separated the dark coloured compounds better from the patatin fraction. High PPO activity in the PI fraction of PFJ did, however, result in low lightness. HIC fractionation could lead to whiter powders, but the additional processing time could also potentially lead to further discoloration by PPO.

Fig. 6 Picture of the different freeze dried powders after fractionation by IEX or HIC. a 2015 patatin; b 2015 PI; c 2014 patatin; d 2014 PI; e 2014 PFJ patatin; f 2014 PFJ PI
Table 8 provides an overall grading from + to ++++ of the measured quality parameters for the IEX and HIC fractions obtained from 2014 PFJ and spray dried powder, with ++++ being most positive for use of the isolate as a food ingredient. This means that a high value is associated with good quality characteristics, i.e. high lightness, low TGA content, low PPO activity, and low total phenolic content. The purification methods did not yield one single sample with a perfect score in all quality parameters, but provides options for different applications for the various isolates. It is seen that e.g. the colour appearance is different for fractions obtained from using either PFJ or spray dried powder. For PFJ the patatin fractions (both IEX and HIC) appear with better colour parameters than PI, which also fits with the observations of lower phenolics in the PFJ patatin. In contrast, for the protein fractions obtained from powder, the best colour parameters were obtained for PI fractions. The total content of polyphenols was higher in the PI than in the patatin
fractions, but surprisingly appeared inverse with colour, and would need further investigation to explain. HIC fractionation generally lowered the PPO activity due to low affinity between PPO and the HIC column, as seen for both PFJ and powder. As TGA was measured only for the 2014 powder, it is not possible to compare this content between PFJ and powder. However, looking at samples derived from 2014 powder, the PI is better than patatin in relation to both TGA and colour parameters, and that further fractionation by HIC does not improve a lot on these parameters for PI. The colour is poorer for patatin compared with PI already after the IEX, but in relation to TGA, though, the subsequent HIC improves TGA, especially for patatin HIC 1, which appears the most promising patatin isolate in terms of the measured quality attributes. For PI the best fractions were PI IEX or PI HIC2. The applications of these fractions as potential food ingredients will depend on further up-scaling of the isolation procedures, as well as on the resulting functional properties of each isolate, in combination with the obtained quality results here.

**Table 8** Overall grading (with +++ most positive) of protein fractions obtained from separation of 2014 PFJ and powder after IEX and HIC, respectively, for the measured quality parameters. Patatin is abbreviated “Pat”, and the spray dried start material “Spray start”.

<table>
<thead>
<tr>
<th></th>
<th>2014 PFJ</th>
<th></th>
<th>2014 Powder</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PI IEX</td>
<td>Pat IEX</td>
<td>PI IEX</td>
</tr>
<tr>
<td></td>
<td>PI HIC 1</td>
<td>PI HIC 2</td>
<td>Pat HIC 1</td>
</tr>
<tr>
<td></td>
<td>Pat HIC 1</td>
<td>Pat HIC 2</td>
<td>Spray start</td>
</tr>
<tr>
<td>TGA</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>Total phenolics</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>PPO activity</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Colour</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

**Conclusion**

Potato proteins were fractionated into a PI rich and a patatin rich fraction by anion exchange chromatography with either PFJ or spray dried powder as source materials. The obtained fractions were further separated by hydrophobic interaction chromatography into two further PI and patatin sub-fractions. IEX purification of re-suspended powder leads to a white PI fraction and a brown patatin fraction, while IEX purification of PFJ can separate the majority of coloured compounds from the patatin fraction. HIC fractionation can improve the colour further, but the increased processing time may also lead to increased discoloration if the fractions are rich in PPO. HIC fractionation significantly lowered the PPO activity due to low affinity between PPO and the HIC column. The total content of polyphenols was higher in the PI than in the patatin fractions, but
surprisingly appeared inverse with colour for spray dried powder. TGA content was below the food limit of 150 ppm in three of four HIC fractions tested and therefore has potential as vegetable based food protein ingredients. The individual glycoalkaloids eluted differently, with chaconine being associated to patatin and solanine to PI proteins. Further work focusing on functional properties of the purified fractions is under progress in order to assess the full potential of potato proteins use as a food ingredient exploiting the functionality potential of these proteins that complies with both vegetarian and vegan diets.

**Acknowledgements**

The authors thank the Future Food Innovation of region Mid-Jutland, Denmark, KMC, AKV Langholt and Aarhus University for financial support of the present work. Furthermore, we thank Ulla Marquardt, laboratory technician at KMC, for analysis of total glycoalkaloids.
References


Paper III

**Gel properties of potato protein isolate and purified fractions by small-scale and large deformation rheology – impact of drying method, protein concentration, pH and ionic strength**

Jesper Malling Schmidt, Henriette Damgaard, Mathias Greve-Poulsen, Anne Vuholm Sunds, Lotte Bach Larsen, Marianne Hammershøj

Manuscript in preparation, intended for publication in Food Hydrocolloids
Gel properties of potato protein isolate by small-scale and large deformation rheology – impact of drying method, protein concentration, pH and ionic strength

In preparation for Food Hydrocolloids

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Highlights

- Drying method of potato protein powder affects gel rheology and texture
- Protein solubility is high at pH 7 and interacts with ionic strength at low pH
- Low pH 3 initiates heat induced gelation at lower temperature than pH 5 and pH 7
- But, gel strength by $G'$ and axial stress is low at pH3 compared to higher pH
- Gels of spray-dried potato protein are more reddish than of freeze-dried protein
Abstract

Potato protein gel properties under different conditions were studied by including two drying methods for a total protein powder (freeze drying and spray drying) and a purified patatin fraction, protein concentrations of 8% and 15% (w/w), range of pH 3-7.5, and 2 ionic strength levels; low (15 mM NaCl) or high (200 mM NaCl). The solubility was highest at pH 7 and 200 mM NaCl, which reached >90%. The thermal protein gelation, recorded rheologically as G’ increase, initiated at lower temperature, when pH and salt promoted conditions of low solubility, i.e. ~22-25 °C compared with neutral pH conditions, where the gelation initiated at 40-50 °C for total protein and 55-60 °C for patatin. Furthermore, an increase in ionic strength from 15 mM NaCl to 200 mM NaCl resulted in a general gelation temperature increase of 5-7 °C for total protein but a decrease for patatin. Gel strength expressed as the storage modulus (G’) generally increased at pH values below or above pH 4.7 for all tested samples. Patatin showed both the lowest and the highest G’ of all tested samples and higher elasticity. Large deformation textural analysis of 15% protein gels showed both pH and ionic strength increase to result in firmer gels, i.e. higher axial stress. For gel elasticity, i.e. Hencky strain, the pH and ionic strength interacted. At 8% potato protein only very weak gels were formed. The gel colour was also affected by pH, as low pH of 5 resulted in more white and yellowish, but less reddish gels. At pH 3.0 and ionic strength of 15 mM could transparent gels be prepared from total protein and patatin and at pH 7 1:15 mM could patatin also produce transparent gels. The water holding capacity of gels only depended on protein concentration.
1. Introduction

The world population growth is booming, which puts a challenge on food security with increasing demand for food protein. This has the recent years lead to exploring of alternative and sustainable protein sources from e.g. plants, algae and insects. One such source may be potato proteins, which are found in a liquid sub-fraction named potato fruit juice “PFJ” during potato starch production. Typically, PFJ contains 2-5% solid matter, of which 35% is N-containing substances, protein, peptides and amino acids (Knorr, Kohler, & Betschart, 1977).

The proteins in PFJ can be classified into three groups. The first group accounting for up to 40% of total protein is the patatin, which are 39-43 kDa glycoproteins (existing as native 80 kDa dimers) with different pI (4.45-5.17) and glycosylation patterns (Baier & Knorr, 2015; Barta, Bartova, Zdrahal, & Sedo, 2012). The second group accounting for ~50% of total protein contains the protease inhibitors (PI) (Pouvreau, et al., 2001). The molecular weight of PIs goes from 4.3-20.6 kDa, with isoelectric point (pI) values of pH 5.1-9.0 (Pouvreau, et al., 2001). The third group is mainly composed of oxidative and other enzymes, like polyphenol oxidase (PPO), lipoxygenase and enzymes associated with starch synthesis (Jorgensen, Stensballe, & Welinder, 2011).

Like other proteins, the potato proteins possess, besides nutritional quality, also promising food functional properties in their ability to form structural networks such as gels, foams (M. Ralet & Gueguen, 2001), and emulsions (Holm & Eriksen, 1980; M. C. Ralet & Gueguen, 2000). In food gels, the proteins are important for both building the gel by binding water in the protein network and the textural properties of the final gel, which is detrimental for the sensory perception of the food gel. The patatin protein has previously proven to readily gel by heat treatment with a relatively low denaturation temperature ($T_d$) of 59-60°C (Creusot, Wierenga, Laus, Giuseppin, & Gruppen, 2011) compared with other gelling proteins such as $\beta$-lactoglobulin, ovalbumin and glycinin. Furthermore, patatin appears to obtain same gel stiffness (measured as storage modulus $G'$) at a much lower protein concentration, i.e. 8% in water (pH 7), than these other proteins where between 11.5-18% protein is required. This is however very dependent on the conditions of ionic
strength, while in presence of 100 mmol/L NaCl the relation is inverted as both β-lactoglobulin and ovalbumin obtain comparable gel stiffness by 7-8% protein as patatin by 9% protein (Creusot, et al., 2011). It is ascribed to the high exposed hydrophobicity of patatin, which at increased ionic strength may decrease its solubility and increases the affinity of the protein to self-aggregation. Another more recent study examining the aggregation behaviour of ovalbumin, β-lactoglobulin and patatin concluded that patatin has a higher tendency to form large and/or dense aggregates compared to the other proteins but it was not easily correlated with the charge and exposed hydrophobicity properties of these proteins (Delahaije, Wierenga, Giuseppin, & Gruppen, 2015). Furthermore patatin forms di- and trimeric structures upon heating which dissociate to monomers in presence of β-mercaptoethanol indicating disulphide bridges (Pots, ten Grotenhuis, Gruppen, Voragen, & de Kruif, 1999). The impact of disulphide bridges in patatin gelation is however not clear.

Research illustrates that the conditions, e.g. pH, ionic strength, temperature, before and during gelation of proteins have significant impact on the gelation process and the final gel properties (Hammershoj, 2001; Rickert, Johnson, & Murphy, 2004; Weijers, Sagis, Veerman, Sperber, & Linden, 2002). Furthermore, due to protein differences between patatin and the protease inhibitors, the studies on patatin gels do not solely illustrate the gel properties of total potato protein.

Besides the protein composition and the gelling conditions, also the pre-treatment during processing of the proteins in consideration may affect gel properties. Especially, heating processes e.g. pasteurisation, spray drying etc. can affect heat sensitive proteins, such as patatin, whey proteins and ovalbumin, to unfold and aggregate and consequently their ability to form gel networks (Hammershoj, Peters, & Andersen, 2004; Lechevalier, et al., 2005; Mishra, Govindasamy-Lucey, & Lucey, 2005). Alternatives to heat processing technologies may be of interest to ensure the protein quality, which for potato proteins is found in usage of high pressure treatment instead of heat treatment to ensure high solubility and improved foam stability (Baier, et al., 2015). As potato protein typically will be spray-dried into a powder for food applications, the
impact of spray-drying on the potato protein for gelling and gel properties is very important to obtain knowledge on.

Even though there has been increased interest in potato proteins recently, only very few studies deal with some aspects of gelling and gel properties of potato proteins protein (Creusot, et al., 2011; Lokra, Helland, Clausen, Straetkvern, & Egelandsdal, 2008).

Our hypothesis is that the potato protein gelling and gel properties depend on protein denaturation, and the pH and ionic strength conditions during gel formation. Hence, the aim of the present study was to evaluate the effect of drying method for total potato protein and the impact of physico-chemical conditions; pH and ionic strength, on protein solubility, protein gelation and final protein gel properties by small- and large-deformation rheology, gel appearance and water holding capacity.

This was studied by two processing technologies for producing dried total potato protein powder from PFJ; freeze-drying = native protein, and spray-drying = denatured protein, in combination with values of pH 3 (≠ pI, i.e. highly soluble patatin and PI), pH 5 (= pI, i.e. insoluble proteins, which may aggregate), and pH 7 (≠ pI of patatin, i.e. soluble; however near pI of some PI proteins, which may aggregate), and low ionic strength (15 mM NaCl) versus high ionic strength (200 mM NaCl).

Potato proteins are purified from PFJ by a vast number of techniques. In laboratory scale, this includes precipitation with acids (hydrochloric acid, sulfuric acid, acetic acid, citric acid), organic solvents (methanol, ethanol, 2-propanol, acetone), metal salts (FeSO₄, FeCl₃, ZnCl₂) (Barta, Hermanova, & Divis, 2008), separation by ion exchange (van Koningsveld, et al., 2001), affinity or hydrophobic chromatography (Racusen, 1989), expanded bed adsorption (EBA) or by ultrafiltration (UF) (Straetkvern & Schwarz, 2012).

UF is a powerful technique for protein concentration and fractionation; problems however exists with membrane fouling due to plant fibers and proteins forming a gel layer on the surface or constricting the pores of the UF membranes (Eriksson & Sivik, 1976; Haberkamp, Ernst, Makdissi, Huck, & Jekel, 2008; Zwijnenberg, et al., 2002). The method of purification and the conditions used will affect the functionality
of the final powder, e.g. EBA will result in native protein with high solubility compared to that of acid precipitated proteins, being denatured (Lokra, et al., 2008).

2. **Materials and Methods**

2.1. **Protein samples**

Two potato protein powders produced from potato fruit juice in December 2014 were provided by KMC, Brande, Denmark. The powders were either spray dried on an Anhydro PSD 55 pilot scale spray dried (SPX FLOW, Soeborg, Denmark) with an inlet temperature of 200 °C and outlet temperature of 70 °C or by freeze-drying in a lab-scale freeze-dryer. The purified fractions were obtained from spray-dried protein isolate from the harvest in December 2014. The purification protocol has been described previously (Schmidt, et al., 2016). The purified fractions were freeze-dried before use.

2.2. **Dry matter determination**

Dry matter of the protein powders was determined by drying ~2 g sample in a HR73 halogen moisture analyzer (Mettler Toledo, Schwerzenbach, Switzerland).

2.3. **Sample preparation**

The protein powders were suspended in various buffers for one hour at r.t. by stirring before use. The used buffers were 30 mM trisodium citrate dihydrate (target pH 3), 320 mM trisodium citrate dehydrate (target pH 3), 22 mM sodium acetate (target pH 5) and 7,5 mM disodium hydrogenphosphate dihydrate (target pH 7) all adjusted to an ionic strength of 15 mM or 200 mM with NaCl. In total, 2 powder drying processes * 3-4 pH values * 2 ionic strengths were applied in the experimental set-up.

The pH of solutions was measured by a PHM 92 pH-meter (Radiometer, Copenhagen, Denmark) and the measured pH summarized in Table 1. Differences were observed between the buffer pH and the actual pH measured, therefore additional samples were prepared with protein suspended in MiliQ water with 15 or
200 mM NaCl and adjusted to pH 3.0 with 1 M HCl. In the following parts of the paper, the pH values referred to correspond to the measured value.

**Table 1.** The pH values of potato protein (spray-dried and freeze-dried) and patatin in protein concentrations of 8% and 15% in respective buffers, n = 2.

<table>
<thead>
<tr>
<th>Used buffer</th>
<th>Spray-dried 8 and 15% solutions</th>
<th>Freeze-dried 8 and 15% solutions</th>
<th>Patatin 8% solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 3 I: 15 mM</td>
<td>4.69 / 4.97</td>
<td>4.99 / 5.01</td>
<td>4.70</td>
</tr>
<tr>
<td>pH 3 I: 200 mM</td>
<td>4.67 / 5.16</td>
<td>4.85 / 4.97</td>
<td>4.90</td>
</tr>
<tr>
<td>pH 5 I: 15 mM</td>
<td>6.14 / 6.30</td>
<td>6.02 / 6.29</td>
<td>Not used</td>
</tr>
<tr>
<td>pH 5 I: 200 mM</td>
<td>6.02 / 6.22</td>
<td>5.93 / 6.30</td>
<td>Not used</td>
</tr>
<tr>
<td>pH 7 I: 15 mM</td>
<td>6.59 / 6.57</td>
<td>6.84 / 6.75</td>
<td>7.52</td>
</tr>
<tr>
<td>pH 7 I: 200 mM</td>
<td>6.56 / 6.43</td>
<td>6.74 / 6.57</td>
<td>7.55</td>
</tr>
</tbody>
</table>

2.4. Relative protein solubility

The powders were suspended in duplicates in the above mentioned buffers to achieve a concentration of 1 g/L corrected for dry matter content. The solutions were stirred one hour following centrifugation at 15300 g for 10 minutes at 4 °C in a tabletop centrifuge (Eppendorf 5417 R, Hamburg, Germany). The supernatant was used for protein determination by the bicinchoninic acid assay (BCA, Thermo Scientific™ Pierce™), with bovine serum albumin (2 mg/mL) as reference protein was used for protein quantification (Smith et al. 1985). Measurements were conducted in triplicates. The relative protein content in percent was calculated based on the amount of protein powder added initially to the buffer. The value is not corrected for ash, or other non-protein impurities present in the powder.

2.5. Dynamic rheological measurements

To follow the gelation process and record the gel strength, suspensions of 8% (w/w) were prepared as described above. 20 ml of sample was transferred to the rheometer (AR G2, TA instruments, New Castle, DE, USA) fitted with a cup-and-bob geometry (cup radius 15 mm, bob radius 14 mm) which was connected to a water-bath providing temperature control during the thermal program. A layer of oil was placed on top
of the solution to prevent evaporation during heating. Strain (0.0001–5 %) and frequency (0.01–100 Hz) sweeps were performed to determine the linear viscoelastic region. Changes in the viscoelastic properties during heating and cooling were measured by the rheometer using oscillatory mode at a frequency of 0.5 Hz and 0.2 % strain. The thermal program started with 5 min equilibration at 20 °C, heating to 85 °C (1 °C/min), holding at 85 °C for 20 min, cooling from 85 °C to 20 °C (1 °C/min), holding at 20 °C for 20 min and finally a strain sweep from 0.1 to 99 %. Storage (G’) and loss (G”) moduli were obtained during the thermal program. Each sample was tested at least in triplicates (patatin in duplicates).

2.6. Preparation of gels for uniaxial compression

Suspensions of 8 and 15 % (w/w) protein were prepared as described previously. The suspension was transferred to a 10 ml plastic syringe (BD Plastipak, REF 302188) with an internal diameter of 14 mm. The inside of the syringe was coated with a thin layer of sunflower oil before transfer of the protein solution. The syringe was heated in a water-bath at 85 °C for 20 min, then cooled in water and stored at 5 °C overnight.

2.7. Gel texture analysis by uniaxial compression.

The syringe was equilibrated for 4 hours to room temperature before careful removal of the gel-cylinder by application of air pressure to one end of the tube. The gel cylinders were cut with an oiled knife to a height of 15 mm. Gel texture was analyzed by uniaxial compression test until fracture of the gel on a FTC TMS-Touch texture analyzer, (Food Technology Corporation, Sterling, USA) with a 25 N load cell, 75 mm diameter flat stainless steel plate and a compression speed of 48 mm/min. Recordings of force (N) and displacement (m) were used in calculation of true axial stress \( \sigma \) (equation 2) and Hencky strain \( \varepsilon \) (equation 3) where \( F = \) force (N), \( A = \) initial end area (m\(^2\)), \( H_i = \) initial gel height (m), and \( H = \) height (m) at fracture.

Each sample was tested at least in triplicates

\[
\sigma = \frac{F H}{A H_i} [Pa]
\]
\[ \varepsilon = - \ln \left( \frac{H}{H_i} \right) \]

2.8. Gel colour

The cut gel cylinders were used for colour measurement with a Minolta Chroma Meter CR-400 (Konica Minolta, Osaka, Japan) using the Hunter Lab scale calibrated against standardized daylight (D65). The L-, a- and b-values reflect lightness (0: black; 100: white), redness (-100: green; 100: red) and yellowness (-100: blue; 100: yellow), respectively. Measurements were conducted in triplicates.

2.9. Water-holding capacity of gels

The water-holding capacity (WHC) of gels was analysed by centrifugation. Small gel pieces were cut and transferred to Amicon Ultra 0.5 mL 10 kDa cut-off centrifugal filters (Merck Millipore Ltd, Tullagreen, Ireland). The mass of the gel piece was noted, before the centrifugal filter was centrifuged at 10000 \( \times \) g for 30 min at 22°C. The expelled water from the gel was collected in a microcentrifuge tube. The water-holding capacity was calculated as shown in equation 4:

\[ WHC = \left( \frac{W_{gel \ after \ centrifugation}}{W_{gel \ before \ centrifugation}} \right) \times 100 \%
\]

2.10. Statistical analysis

The results are presented as means with standard deviations. Significant differences between treatments were determined by two-way ANOVA analysis with parameters pH, ion strength and interactions hereof using GraphPad Prism 6 (GraphPad Software Inc, La Jolla, USA, version 6.01). Differences were regarded to be significant at minimum 95 %-level (P < 0.05).
3. Results

3.1. Protein solubility

The solubility of the different protein samples were tested at pH 3, 5 and 7 at ionic strength of 15 and 200 mM. The freeze-dried (Fig. 1A) and spray-dried (Fig. 1B) total protein isolate showed similar solubility profiles at low ionic strength with high solubility at pH 3 and pH 7 and a minimum at pH 5. Increase in ionic strength to 200 mM resulted in a 17% (point) increase in solubility at pH 5 for both powders, but a decrease of 18% for the spray-dried powder at pH 3. Overall, the highest solubility for both powders was obtained at pH 7 and 200 mM NaCl, which reached >90%.

![Fig. 1 Relative protein solubility at pH 3, 5 and 7 at ionic strength of 15 or 200 mM. A) Freeze-dried total protein isolate; B) Spray-dried total protein isolate.](image)

3.2. Gelation profile by dynamic rheological measurements

Small deformation dynamic rheology was applied to monitor heat-induced gelation during the 20-85 °C temperature ramp of the 8 % (w/w) solutions. Liquid solutions have low G' values initially until a certain temperature, where a rapid increase in G' is observed i.e. the gel point (Frydenberg, Hammershoj, Andersen, Greve, & Wiking, 2016). Results for spray-dried protein isolate at an ionic strength of 15 and 200 mM are presented in Fig. 2A and 2B, respectively. At an ionic strength of 15 mM, the pH 4.69 sample had at a markedly different profile than the other samples with an initial 10-fold higher G' at 20 °C. An increase in
G’ for total protein at pH 3.0, pH 6.14 and pH 6.59 was seen at ~54, ~33 and ~40 °C respectively (Fig. 2A). Increase in ionic strength from 15 mM to 200 mM resulted in markedly different profiles for the pH 3.0 sample, showing build-up of structure already from 22 °C instead of 54 °C. The initial rise in G’ was increased to ~40 °C and ~47 °C for pH 6.02 and pH 6.56, respectively, i.e. temperatures were 5-7 °C higher for gel point at high ionic strength. For the samples with ionic strength of 15 mM, clear differences due to pH were seen in the final G’ at 85 °C, with values of 320, 105, 238 and 460 Pa for pH 3.0, pH 4.69, pH 6.14 and pH 6.59, respectively. At an ionic strength of 200 mM the pH 6.02 and pH 6.56 samples were more alike with values of 370 and 425 Pa, while pH 4.67 reached a G’ of 200 Pa. The pH 3.0 sample had a substantially higher G’ value of ~1200 Pa. The profiles for the freeze-dried samples were comparable to the spray-dried samples except for pH 6.02 at I: 15 mM, which had an initial increase in G’ at 22 °C, i.e. 10 °C lower than the spray-dried sample (results not shown).

Heating profiles of 8 % patatin at an ionic strength of 15 mM are shown in Fig. 2C. The sample at pH 4.7 had a G’ of 16 Pa at 20 °C, which decreased until at temperature of 52 °C, after which it increased again. Gel points for pH 3.0 and pH 7.52 were at 50 and 62 °C, respectively. The final G’-values at 85 °C were 845, 104 and 790 Pa. For patatin at an ionic strength of 200 mM (Fig. 2D) both the sample at pH 3.0 and pH 4.9 had structure recorded as G’ values >10 Pa at 20 °C, while the sample at pH 7.55 had a clear gelation point at 55 °C. The G’-values at 85 °C were 370, 500 and 1110 Pa, for pH 3.0, pH 4.9 and pH 7.55, respectively.

3.3. Gel properties by dynamic rheological measurements

Following the heating ramp from 20-85 °C, samples were cooled to 20 °C and the gel strength expressed as the storage modulus (G’). A strain sweep was conducted from 0.2 to 99 % and the resulting loss in G’ monitored. When strain is increased from 0.2-99 %, the linear viscoelastic region (LWR) will eventually be surpassed. The strain causing a 10 % reduction of the initial G’ was defined as the end of LWR and used to compare the different samples. A low strain value indicates that the sample cannot withstand much deformation before structural breakdown occurs.
Fig. 2 Gelation profiles measured by oscillatory rheology during heating from 20-85 °C of 8 % (w/w) spray-dried protein isolate at ionic strength A) 15 mM; B) 200 mM, and 8 % (w/w) patatin at ionic strength C) 15 mM; D) 200 mM.

The storage modulus (G’) of freeze- and spray-dried total protein isolate was affected by pH and ionic strength changes (Fig. 3). At an ionic strength of 15 mM, G’ was lowest at pH 4.7-4.9 with higher values at pH 3, and increasingly higher values at pH ~6.1, and at pH~6.8 yielding the strongest gel. At I: 200 mM, G’ was lowest at pH 4.7-4.9 and less effect was seen upon pH increase. For pH 3.0, the spray-dried sample had a markedly higher G’ than the freeze-dried sample. Generally, did an increase in ionic strength result in higher G’ except at the highest pH (Fig. 3A, B). Spray-drying resulted in higher gel strength values compared to freeze-drying at all pH and ionic strength conditions. Ionic strength and pH had a different impact on
purified patatin compared with the total protein isolate, with a marked decrease in G’ at high ionic strength at pH 3, while an increase was seen at the other pH values (Fig. 3C).

For both drying methods, pH 3.0 at I:200 resulted in the lowest strain values and pH 3.0 at I: 15 the highest. A trend was observed at the other pH values with the highest ionic strength resulting in the highest strain. For patatin, markedly higher strain values were observed at pH ~7.5 with an ionic strength of 200 mM resulting in the highest values measured. At pH 3.0 the patatin sample behaved more alike to the total protein powder.

**Fig. 3** Storage modulus (G’) and strain value causing 10 percent reduction of the initial G’ of 8% (w/w) total protein isolate or patatin gels at different pH values at ionic strength 15 or 200 mM. All data are recorded after the completion of the temperature program and measured at 20 °C at a frequency of 0.5 Hz and 0.2% strain. A) G’ of freeze dried potato protein; B) G’ of spray dried potato protein; C) G’ of patatin; D) strain freeze dried; E) strain spray dried; F) strain patatin. Different letters in the same figure indicate significant different samples at P<0.05.
3.4. Gel properties by uniaxial compression

The 8 and 15% (w/w) gels were prepared for uniaxial compression analysis, to assess gel texture at large strain conditions. The true axial stress \(\sigma\) of 15% (w/w) freeze- and spray-dried total protein gels were significantly affected by pH and ionic strength, with a low strength at pH \(\sim 5\) and an increased gel texture for I: 200 mM compared to I: 15 mM at all pH values (Fig. 4A, B). Spray drying of the protein did again result in a stronger gel compared to freeze-drying. The 8% (w/w) gels of spray-dried protein resulted in equal stress of pH 6.0 I: 200 mM and pH 6.6 I: 15 mM, but a significantly harder gel at pH 6.6 I: 200 mM (Fig. 4C).

![Graphs](image)

**Fig. 4** Textural evaluation of 15 and 8% (w/w) total protein isolate gels measured by uniaxial compression expressed as stress and strain. A) Stress of freeze-dried 15% (w/w); B) spray dried 15% (w/w); C) spray dried 8% (w/w); D) Strain of freeze-dried 15% (w/w); E) spray-dried 15% (w/w), F) spray-dried 8% (w/w). Different letters in the same figure indicate significant different samples at \(P<0.05\).

Hencky strain \(\varepsilon\) of 15% (w/w) freeze-dried and spray-dried total protein isolate gels were significantly affected by pH \((P<0.001)\) but not ionic strength (Fig. 4D, E). Both powders showed a similar trend with low...
strain at pH ~5 and higher strain at increasing pH. For 8% (w/w) spray dried gels, pH 6.0: 200 mM and pH 6.6: 200 mM resulted in similar high strain values, with pH 6.6: 15 mM yielding a significantly lower strain than the two other conditions, meaning this gel was less compressible when reaching its point of fracture.

The drying method had only little impact on the strain.

**Table 2.** Colour of 15% (w/w) total potato protein isolate gels evaluated by lightness $L$ (0 = black, 100 = white), redness $a$ (-100 = green, 100 = red), and yellowness $b$ (-100 = blue, 100 = yellow). Different letters in the same column indicate significant different samples at $P<0.05$.

<table>
<thead>
<tr>
<th>Drying method</th>
<th>pH and ionic strength (I)</th>
<th>$L$</th>
<th>$a$</th>
<th>$b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spray-dried</td>
<td>pH 4.9:15</td>
<td>62.20 ± 0.13 $^A$</td>
<td>1.27 ± 0.01 $^A$</td>
<td>5.94 ± 0.01 $^A$</td>
</tr>
<tr>
<td>Freeze-dried</td>
<td>pH 5.0:15</td>
<td>64.13 ± 0.03 $^B$</td>
<td>0.64 ± 0.01 $^E$</td>
<td>5.53 ± 0.01 $^B$</td>
</tr>
<tr>
<td>Spray-dried</td>
<td>pH 5.16:200</td>
<td>63.57 ± 0.09 $^C$</td>
<td>1.21 ± 0.02 $^A$</td>
<td>5.54 ± 0.02 $^B$</td>
</tr>
<tr>
<td>Freeze-dried</td>
<td>pH 4.97:200</td>
<td>63.92 ± 0.14 $^{BC}$</td>
<td>0.56 ± 0.01 $^C$</td>
<td>5.20 ± 0.01 $^B$</td>
</tr>
<tr>
<td>Spray-dried</td>
<td>pH 6.30:15</td>
<td>54.14 ± 0.05 $^B$</td>
<td>4.28 ± 0.02 $^D$</td>
<td>4.32 ± 0.01 $^D$</td>
</tr>
<tr>
<td>Freeze-dried</td>
<td>pH 6.29:15</td>
<td>52.45 ± 0.23 $^E$</td>
<td>2.70 ± 0.01 $^F$</td>
<td>2.96 ± 0.03 $^E$</td>
</tr>
<tr>
<td>Spray-dried</td>
<td>pH 6.22:200</td>
<td>55.29 ± 0.05 $^E$</td>
<td>4.26 ± 0.03 $^B$</td>
<td>3.75 ± 0.01 $^F$</td>
</tr>
<tr>
<td>Freeze-dried</td>
<td>pH 6.30:200</td>
<td>54.68 ± 0.05 $^D$</td>
<td>2.68 ± 0.03 $^E$</td>
<td>2.46 ± 0.01 $^G$</td>
</tr>
<tr>
<td>Spray-dried</td>
<td>pH 6.57:15</td>
<td>53.50 ± 0.02 $^G$</td>
<td>4.47 ± 0.02 $^F$</td>
<td>3.82 ± 0.01 $^F$</td>
</tr>
<tr>
<td>Freeze-dried</td>
<td>pH 6.75:15</td>
<td>50.38 ± 0.18 $^H$</td>
<td>2.94 ± 0.01 $^G$</td>
<td>2.85 ± 0.04 $^H$</td>
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<tr>
<td>Spray-dried</td>
<td>pH 6.43:200</td>
<td>54.27 ± 0.01 $^D$</td>
<td>4.52 ± 0.01 $^E$</td>
<td>3.60 ± 0.01 $^I$</td>
</tr>
<tr>
<td>Freeze-dried</td>
<td>pH 6.57:200</td>
<td>52.65 ± 0.35 $^E$</td>
<td>2.98 ± 0.02 $^G$</td>
<td>2.56 ± 0.08 $^G$</td>
</tr>
</tbody>
</table>

3.5. Colour and visual appearance of gels

The visual appearance of 15% (w/w) total isolate gels were highly affected by pH, with pH ~5 yielding gels with significantly more light gels (higher $L$), less reddish (lower $a$) and more yellowish (higher $b$) than higher pH (Table 2). The ionic strength did not play a significant role on the gel colour. The biggest effect on drying method is seen in the $a$-values, with spray-dried samples having a significantly more reddish colour than freeze-dried ($P<0.05$). All gels were highly opaque regardless of pH, ionic strength or drying method. The 8
% (w/w) gels used for rheology were not measured by the chromameter, however the gels were assessed visually. Spray dried powder at pH 3.0 and I: 15 mM resulted in a transparent gel, while at pH 3 and I: 200 mM and all other pH and salt conditions gave opaque gels. For patatin, did pH 3.0 at I: 15 mM and pH 7.5 at I: 15 mM result in transparent gels, and the remaining conditions in opaque gels. Spray-dried powder was adjusted to pH 7.5 at I: 15 mM but the resulting gel was opaque in contrast to patatin at the same conditions.

3.6. Water-holding capacity of gels

Water-holding capacity (WHC) of gels prepared for uniaxial compression were tested by centrifugation and the results are shown in Fig. 5. For 15 % (w/w) total protein isolate gels no significant differences were seen between pH and ionic strength, and both drying conditions resulted in WHCs between 45-50 %. The 8 % (w/w) spray-dried total isolate gels had a much lower WHC (30-34 %) compared to 15 % (w/w) gels.

Fig. 5 Water holding capacity (WHC) of total potato protein isolate gels pH 3, 5, 7 and I: 15 and 200 mM. A) 15 % (w/w) freeze-dried; B) 15 % (w/w) spray-dried; C) 8 % w/w spray dried. Different letters within same chart indicate significantly different samples.
4. Discussion

4.1. Protein solubility

The solubility data of total protein isolate samples agrees with the data of (van Koningsveld, et al., 2001) with high solubility at pH 3 and pH 7 and a minimum from pH 4-5. Spray-drying did not result in inferior solubility compared to freeze-drying except at pH 3 and pH 4 at I: 200 mM. The drying method can however result in very different solubility as reported by (Lokra, et al., 2008) with 10% solubility, when spray-drying with outlet temperature of 165 °C was applied, compared to ~70% solubility of vacuum freeze-dried protein.

4.2. Gelation profile by dynamic rheological measurements

The gelation profile of spray-dried total protein isolate was affected by both pH and ionic strength, with a higher ionic strength increasing the gelation temperature at pH 6 and pH ~6.6. Differential Scanning Calorimetry studies have also shown increasing denaturation temperature (T_d) when going from I: 15 mM to I: 200 mM at pH 7 (van Koningsveld, et al., 2001). Strikingly high G' values below 25 °C were observed for spray-dried powder at pH ~4.7, patatin at pH 4.7 and I: 15 mM and patatin at pH 3.0 and I: 200 mM. These high values is believed to be caused by suspended undissolved protein particles since the observed conditions fits nicely to solubility minimum as reported in this paper and previously (van Koningsveld, et al., 2001).

The gel points (a sudden steep increase in G') were unusually low (< 48 °C) for all spray-dried samples, when compared to the denaturation temperature (T_d) of PFJ proteins at pH 7 of 66 °C (van Koningsveld, et al., 2001). One would assume that denaturation should precede aggregation, but other authors have reported similar behaviour i.e. an increase in G' at a lower temperature than (T_d) (Stading & Hermansson, 1990). Furthermore, experiments have shown that heating above 30 °C during drying of PFJ results in significantly lower re-solubility (especially for patatin), which indicates that protein-protein interactions are happening already at these low temperatures (Bartova & Barta, 2008). For patatin, low gelation
temperatures were found at pH 3.0 and pH 4.9, while pH 7.5 gave values from 55-60 °C in line with previously published (Creusot, et al., 2011).

Striking differences were found for the value of storage modulus at 85 °C, with the spray dried powder at pH 3.0 and I: 15 mM and patatin at pH 7.5 and I: 15 yielding values of > 1100 Pa, while spray dried powder and patatin had values of ~10 Pa at pH 4.7 and I: 15 mM. A high G’ at high temperature may be important in some applications, where structure and suspension powder is needed during heating.

4.3. Gel properties by small- and large-scale deformation and visual appearance of gels

Total potato protein isolate of either spray or freeze-dried origin produced the weakest gels at pH ~4.7-5 and stronger gels below and above this pH. For patatin, the weakest gels were formed at conditions that correspond to reported solubility minimums. This suggests a relationship between protein solubility and gel strength for the tested proteins, however, the spray dried sample at pH 3.0 and I: 200 mM showed high gel strength even though solubility was decreased compared to at pH 3 and I: 15 mM. Globular proteins tend to form particulate (opaque) gels when pH = pI or at high ionic strength and fine-stranded (translucent) gels when pI < pH < pI or low ionic strength (Foegeding, 2006). As an example, egg albumen proteins cover a range of pI from pH 4.1-6.6 for proteins representing >80% of the quantity, and gels prepared at pH 5, i.e. near pI are white and turbid, which become more grey at neutral pH 7, and are completely transparent at highly alkaline conditions at pH 11, i.e. pH >> pI (Hammershoj, 2001). As stated in the introduction, patatin has a pI of 4.45-5.17, while the protease inhibitors have pI of 5.1-9.0. Hence, a fine-stranded gel would therefore be expected at pH 3 and I: 15 mM, and the translucent gels of both patatin and spray dried powder agrees with this theory. Patatin could also form a translucent gel at pH 7.5, while spray dried powder formed an opaque gel, most likely due to presence of protease inhibitor proteins with pI in this region.

The gels prepared with patatin showed higher response to changes in pH and ionic strength yielding either very strong or very weak gels. By having mixtures of proteins with different pI values, like in the spray dried
powder, a solubility minimum and hence low gels strength can be mitigated by having other proteins with higher solubility, thus providing gels with more uniform performance.

The storage modulus (G’) of 8 % (w/w) gels increased when going from I: 15 mM to 200 mM for the total isolate samples at pH 4.7 and pH ~6.1 but decreased at pH ~6.6. In contrast, did results from the strain sweep show an increase strain-value for all samples, when ionic strength was increased. The behaviour at pH 6.6 and I: 200 mM with decreased G’ and increased strain suggests another gel network compared to the other conditions tested. Gels at pH 3.0 also behaved differently with conditions giving the highest G’ yielding the lowest strain values, again suggesting another type of network.

Dependent on the specific protein, a salting-in or salting-out effect by altering the ionic strength may then influence the gel texture of the protein, which for e.g. egg albumen gels was significantly higher in Hencky strain at ~5mM NaCl than below or above, while the textual axial stress was unaffected (Hammershoj, 2001), furthermore will gel hardness and stiffness usually increase with ionic strength until a given optimum concentration is reached, and above this optimum the gel becomes softer (Doi, 1993). Axial stress of 8 % (w/w) gels did not decrease at pH 6.6 I: 200 mM as seen for G’ during rheometry measurements. Correlation between small-deformation rheometry and large-deformation uniaxial compression data of 8 % (w/w) gels were made indicating a high correlation ($R^2 = 0.88$) between the strain leading to 10 % reduction in G’ and Hencky strain, but no correlation between G’ and axial stress ($R^2 = 0.06$).

Axial stress increased as a function of both pH and ionic strength with high pH and high salt yielding the strongest 15 % (w/w) total isolate gels. Hencky strain was similar at pH ~6.3 and ~6.7 while pH 5 had significantly lower values, again indicating a different gel-network structure at this pH.

Spray-drying resulted in higher textural strength both measured as G’ by small-deformation rheometry and as axial stress at fracture of gels by large-deformation in uniaxial compression compared with freeze drying. The differences were in the range of ~500-2300 Pa for G’ and ~4 kPa for axial stress. It is believed that spray drying results in limited denaturation, partial protein unfolding and thus a higher surface hydrophobicity,
which may increase the protein gel firmness by increased hydrophobic interaction in the gel network (Hammershoj, Rasmussen, Carstens, & Pedersen, 2006). However, if spray-drying is done below denaturing conditions there may be no difference between the functional properties of e.g. ovalbumin due to drying method (Kitabatake, Indo, & Doi, 1989), although the drying of protein can change the properties compared with native protein (non-dried). It can be difficult to predict the effect of drying process on protein functionality in food relations of two overall reasons. First, different proteins differ in sensitivity to drying, and second, dependent on the kind of change and magnitude of change in protein structure upon drying it may provide a change in one functional property but not in another (Luck, et al., 2013). The observed effect on increased gel strength by spray drying indicated a change in the potato protein conformation, which do require a certain heat load. In contrast, for the soy-protein fractions p-conglycinin and glycinin the opposite relation is found, as spray-drying with increased temperature results in protein gels with lower G’ values and being more fragile (Tarone, Fasolin, Perrechil, Hubinger, & da Cunha, 2013). Furthermore, in a previous study on heat induced potato protein gels of freeze-dried and spray dried protein at 95 g/L the gel from freeze-dried protein had a complex modulus, G* of 1.4 kPa after cooling, and no gelling occurred from the spray-dried potato protein (Lokra, et al., 2008). Hereby, indicating quite weaker gel properties compared to the present potato protein regardless of drying method. The present gel colour results also indicated that spray-drying had been more severe than freeze-drying, while both a-values (redness) and b-values (yellowness) were significantly higher for all gels prepared from spray dried potato protein compared to gels prepared from freeze-dried protein at equal pH and ionic strength conditions.

4.4. Water holding capacity of gels

No significant differences were found in WHC of 15 % (w/w) total isolate gels of pH or ionic strength. 8 % (w/w) gels had lower WHC than 15 % (w/w) gels and pH 6 I: 200 mM had lower WHC than pH ~6.6 I 15 and 200 mM. Gel stiffness has been correlated to WHC with softer gels having lower WHC (Nieuwland,
Bouwman, Povreau, Martin, & de Jongh, 2016). This relationship was however not found for 8 % (w/w) total isolate gels since pH 6: 200 had higher γ(%) and Hencky strain than pH 6.6: 15 gels.

5. Conclusion

In this study gels were prepared from either spray-dried or freeze-dried potato total protein powder or from purified patatin. Protein concentration of 8 % (w/w) produced gels that were used for oscillatory rheology to map gelation temperature, storage modulus and deformation properties. For some of the samples, structure was present in the protein dispersions before heating. These viscous samples were associated to pH and ionic strength conditions that favoured low protein solubility. Conditions that resulted in poor solubility generally resulted in low gel strength. The strength of gels for total protein powder were generally enhanced at an ionic strength of 200 mM compared to 15 mM, and optimum pH conditions were below or above pH ~4.7. Spray-dried powder gave stronger gels than freeze-dried powder in all tested conditions. Gels made of patatin resulted in both the lowest and the highest $G'$-values of all samples indicating that test conditions had higher impact on the purified protein than for gels of a mixture of proteins. Patatin gels at high pH showed remarkably higher resistance to deformation that total protein gels.

The 8 % and 15 % (w/w) gels were used for large deformation studies. Increased pH and salt resulted in gels with highest stress. Strain was significantly lower at pH 5 compared to higher pH values. A correlation was found between strain measured by rheometry and large deformation studies of 8 % (w/w) gels.

Opaque gels indicating particulate gels were found at most pH and salt conditions except at pH 3.0 and I: 15 mM for total isolate powder, and at pH 3.0 and pH 7.5 at I: 15 mM for patatin indicating either particulate or fine-stranded gels. The water holding capacity of 15 % (w/w) protein gels did not differ for the tested conditions (pH 5-6.7, I: 15 mM and 200 mM) indicating that the same type of particulate network was produced at these conditions.
This work shows that spray-drying do not diminish the gel forming ability of total protein powder, and gives further insight into the impact of pH and salt on potato protein. Patatin may, depending on pH and salt condition, show increased gel strength, clarity and elasticity compared to total protein isolate and this may be important for different food applications.

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References


Paper IV

Foam and emulsion properties of potato protein isolate and purified fractions

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In preparation for Food Hydrocolloids

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Highlights

- Foam and emulsions of 7 different potato protein fractions were compared
- Proteins were separated by ion exchange and hydrophobic interaction chromatography
- Patatin had superior foam performance at pH 3, protease inhibitors at pH 5 and pH 7
- Patatin had higher emulsion stability and viscosity than protease inhibitors
- The most hydrophobic patatin and protease inhibitors had the best emulsions

Keywords: potato; protein: patatin; foam; emulsion; surface tension
Abstract

Spray dried potato protein and specific isolated fractions were used for foaming and emulsification studies. The spray dried protein was separated into a patatin and a protease inhibitor (PI) rich fraction by ion exchange chromatography (IEX), and these two fractions were purified by hydrophobic interaction chromatography into a low (HIC 1) and a high (HIC 2) hydrophobic fraction. Foam overrun for the spray dried powder and all patatin fractions were highest at pH 3, with gradually lower values at pH 5 and 7, while the PI fractions had highest overrun at pH 5 and equally lower values at pH 3 and 7. Relative foam stability was varying from 18-78 % of the initial foam at pH 3 while lower variation of 67-80 % was seen at pH 5 and 7. The HIC fractions did generally perform better than the spray dried powder and IEX fractions, with patatin HIC 1 and 2 showing superior performance at pH 3 and PI HIC 2 at pH 5 and 7. Emulsions were characterized by emulsion stability and activity, emulsion droplet size and small scale dynamic rheological measurements. The PI, and especially PI HIC 1 showed bad emulsion properties with low stability, large droplet size and thinner texture. Interestingly PI HIC 2 showed much better emulsion properties on par with the spray dried powder and patatin, while showing more frequency dependent textural response. Overall, the best emulsion could be made with patatin HIC 2, thus showing the importance of hydrophobicity for protein functionality.
1. Introduction

Potato protein has in recent years drawn more and more attention as a protein source for human consumption and as a unique food ingredient. Industrially potato starch is the main value-added ingredient from potatoes with the proteins being located in a side stream from starch production known as potato fruit juice (PFJ). PFJ contains roughly 2-5 % solids of which 35 % is N-containing substances i.e. protein, peptides and amino acids (Knorr, Kohler, & Betschart, 1977). The potato proteins have been concentrated from PFJ by acid precipitation, but to preserve the functional properties more gentle techniques needs to be used e.g. expanded bed absorption (Lokra, Helland, Claussen, Straetkvern, & Egelandsdal, 2008), ion exchange chromatography (van Koningsveld, et al., 2001) or ultrafiltration (Straetkvern & Schwarz, 2012).

The potato proteins can roughly be divided into three main groups, with the first being the patatins, representing up to 40 % of the total protein. The patatins are glycoproteins with a molecular weight of 39-43 kDa (existing as native 80 kDa dimers) with different pl (4.45-5.17) and glycosylation patterns (1-3 glycosylations) (Barta, Bartova, Zdrahal, & Sedo, 2012). The second group of potato proteins is the protease inhibitors (PI), which represents up to 50 % of the total protein and is this groups is further divided into seven sub-groups (Pouvreau, et al., 2001). The molecular weight of the PI proteins varies widely, from 4.3-20.6 kDa, with pl values of 5.1-9.0 (Pouvreau, et al., 2001). The last group of proteins is mainly composed of oxidative enzymes, like PPO, lipoxygenase and enzymes associated with starch synthesis (Jorgensen, Stensballe, & Welinder, 2011).

Two important functional properties of food proteins are the ability to form and stabilize foams and emulsions by adsorbing to the interface between air-water or water-oil, hence lowering the interfacial tension and providing electrostatic and steric stabilisation (Wierenga & Gruppen, 2010). Only limited results have been published on foam and emulsification behavior of potato proteins and even fewer on the purified fractions (Ralet & Gueguen, 2000, 2001; van Koningsveld, et al., 2002; van Koningsveld, et al., 2006).
The aim of this study was therefore to provide further insight into foam and emulsion properties of potato proteins and purified fractions thereof. It was theorized that fractionation of a total protein isolate by first ion exchange and then hydrophobic interaction chromatography will lead to superior functional properties.

2. Materials and Methods

2.1. Protein samples

Spray dried potato protein isolate powders from the harvest 2015 were provided by KMC, Brande, Denmark. Fractionation of the protein isolate by ion exchange chromatography results in a patatin fraction (Pat) and a protease inhibitor fraction (PI), further purification by hydrophobic interaction chromatography (HIC) resulted in two patatin fractions (Patatin HIC 1 and Patatin HIC 2) and two protease inhibitor fractions (PI HIC 1 and PI HIC 2). Fractionation was done as described previously (Schmidt, et al., 2016). The purified fractions were concentrated and diafiltered against distilled water on an Ultraglab tangential flow system with a 10 kDa Minimate™ membrane (Pall, New York, USA) and freeze-dried before use.

2.2. Preparation of protein dispersions

The freeze-dried powders were suspended in buffer for one hour before use. The used buffers were 30 mM trisodium citrate dihydrate (target pH 3), 22 mM sodium acetate (target pH 5) and 7.5 mM disodium hydrogenphosphate dihydrate (target pH 7) all adjusted to an ionic strength of 50 mM with NaCl. For emulsions at pH 3 a 95 mM trisodium citrate dihydrate was used. Prior to foam or emulsion preparation suspensions were centrifuged at (4700 rpm, 15 min, 4 °C) in order to avoid any insolubilized material in the sample for analysis.
2.3. SDS-PAGE

SDS-PAGE using Criterion™ TGX™ 8-16 % precast gels (Bio-Rad, Richmond, CA, USA) was performed essentially as described by (Laemmli, 1970). The samples were mixed 1:1 with sample buffer (20 mM Tris, 2 % SDS, 20 % glycerol, pyronin Y), and reduced with 1/10 vol 0.2 M DTE and boiled for 3 min. 30 µL sample of 1 mg/mL were loaded onto the gel. Gels were stained with Coomassie Brilliant blue G-250. Molecular mass was estimated by a prestained broad range molecular weight marker (Thermo Scientific™ Spectra™ Multicolor Broad Range Protein Ladders).

2.4. Protein concentration determination

The bicinchoninic acid assay (BCA, Thermo Scientific™ Pierce™), with bovine serum albumin (2 mg/mL) as reference protein was used for protein determination. Measurements were conducted in duplicates.

2.5. Zeta-potential

Zeta-potential (mV) of 10 mL protein solutions at a concentration of 1 g L⁻¹ and at pH 3, 5 and 7 were determined by a Stabino® (Particle Metrix, Meerbusch, Germany) fitted with a 200 µm piston by measuring for 180 seconds and the endpoint recorded. A minimum of four measurements per sample were made.

2.6. Foam analysis

Foam was formed by shaking 20 mL of 1 g L⁻¹ solution in 100 mL closed cylinders at 4 Hz for 45 seconds. Foam overrun (FO) were calculated (equation 1) as a relative overrun volume based on $V_{\text{foam}} = \text{volume of foam (L)}$ and $V_{\text{liquid}} = \text{volume of liquid (L)}$. The total volume of foam and liquid as well as the liquid volume
were recorded for two hours. The foam stability was calculated (equation 2) as the relative foam volume (FV) (Hammershoj, Peters, & Andersen, 2004). Minimum three repetitions were made per sample.

\[ FO = \frac{V_{foam}}{V_{liquid}} \text{[L/L]} \]  
\[ FV = \frac{V_{foam \ t=2h}}{V_{foam \ t=0h}} \times 100 \% \]

2.7. Surface tension

Surface tension was determined using a platinum Wilhelmy plate (19.62 × 10 × 0.1 mm) controlled by a Sigma 700 force tensiometer (Biolin Scientific AB, Västra Frölunda, Sweden) with the following measuring conditions; 6 mm wetting depth, 4 s stabilization time, 4 s integration time, 20 mm/min pull speed.

To a small glass vessel filled with 38 mL buffer, 2 mL of 10 g L\(^{-1}\) protein solution resulting in a final protein concentration of 0.5 g L\(^{-1}\) was applied to the bottom with a syringe and the measurements started. The initial surface tensions of the buffers were checked to be ~72 mN/m (as for pure water) indicating no surface active compounds present before adding the protein samples. The decrease in surface tension was monitored for six hours and the resulting surface tension \(\gamma\) (N m\(^{-1}\)) value recorded. Data for the first three hours was fitted to an exponential one-phase decay function and the half-life calculated as the time for surface tension to decrease to reach 50% of the total decrease, i.e. the lower half-life time, the faster initial decrease in surface tension, and the higher surface activity of the actual sample. Measurements were done in triplicate.

2.8. Interfacial tension

Interfacial tension was measured at the oil-water interface between 1 g L\(^{-1}\) protein solution (pH 3) and sunflower oil using a Wilhelmy plate (as described in section 2.6.) for nine hours with the following
measurement conditions; 14 mm wetting depth, 4 s stabilization time, 4 s integration time, 20 mm/min pull speed. Measurements were done in triplicate.

2.9. Emulsion preparation

Emulsions were prepared by mixing 6 ml 20 g L\(^{-1}\) protein solution (pH 3) with 18 ml sunflower oil by an ultra turrax T25 (IKA Werke GmbH & Co. KG, Staufen, Germany) for 5 min at 8000 rpm followed by 4 min at 13500 RPM. Two emulsions were prepared per protein sample.

The emulsifying activity index (EAI) (equation 3) and emulsifying stability index (ESI) (equation 4) were determined by the method originally developed by Pearce and Kinsella (1978) with later modifications (Cheung, Wanasundara, & Nickerson, 2014) using UV absorbance at \(\lambda = 500\) nm. \(A_0\) is absorbance of the diluted sample at time \(= 0\) and \(A_{10}\) at time \(= 10\) minutes, \(N\) is a dilution factor (500), \(c\) is protein concentration (0.02 g/mL) and \(\varphi\) is the oil volume fraction (0.75). The emulsion was diluted 500 times in 0.1 % SDS in two steps by diluting 1 g emulsion in 100 mL 0.1% SDS, and 1 mL hereof was further diluted 5-fold in 0.1% SDS. After mixing for 30 s, the diluted emulsion was measured in a plastic cuvette at 500 nm in a UV-visible spectrophotometer.

\[
EAI \left( \frac{m^2}{g} \right) = \frac{2 \times 2.303 \times A_0 \times N}{c \times \varphi \times 10000}
\]  

\[
ESI \ (min) = \frac{A_0}{A_0 - A_{10}} \times t
\]

2.10. Measurement of emulsions droplet size

Droplet size distribution of emulsions was measured by a Mastersizer 2000 (Malvern Instruments, Worcestershire, U.K.) with a Hydro 2000S sample unit. Emulsions were diluted 1:2 (v:v) with 1 % SDS and volume-weighted mean diameter (D4,3) and surface-weighted mean diameter (D3,2) were determined from each emulsion, resulting in a total of six measurements per sample.

2.11. Dynamic rheological analysis of emulsions
Emulsion was transferred to the rheometer (AR G2, TA instruments, New Castle, DE, USA) fitted with a parallel plate-plate geometry with a gap of 1 mm (plate radius 40 mm). After a 10 min equilibration oscillatory frequency sweeps (0.01–50 Hz) at 0.1 % strain and strain sweeps (0.01-10 %) at 0.5 Hz were performed at 20 °C. Two frequency sweeps were performed per emulsion resulting in four measurements, except for the PI HIC 1 fraction where only one sample was available.

2.12. Statistics
The results are presented as means with standard deviations. Significant differences between treatments were determined by two-way ANOVA analysis with parameters of protein fraction and pH and interactions hereof using GraphPad Prism 6 (GraphPad Software Inc, La Jolla, USA, version 6.01). Differences were regarded to be significant at mimimum 95 %-level ($P < 0.05$).
3. Results and Discussion

3.1. Protein composition

The composition of the protein samples by SDS-PAGE are visualized in Figure 1.

![Figure 1. SDS-PAGE gel of protein samples. Lane 1, molecular weight marker with protein masses (Mw) in kDa to the left of the gel; Lane 2, potato protein powder; Lane 3, Patatin HIC 1; Lane 4, Patatin HIC 2; Lane 5, PI HIC 1; Lane 6, PI HIC 2.](image)

From Figure 1 it is obvious that the protein bands with masses of ~40-43 kDa dominate the patatin HIC 1 and HIC 2 fractions with Patatin HIC 2 being enriched with a higher molecular weight isoform of patatin. The PI fractions are dominated by proteins with molecular weight of 10-24 kDa for PI HIC 1 and 14-20 kDa for PI HIC 2 respectively.

3.2. Zeta-potential

The zeta-potential was measured of the spray dried powder, the patatin fractions and the PI fractions at three different pH values (Figure 2). The zeta-potential gives the surface charge of the respective proteins.
in the sample. The ionization of the protein, which depends on the actual pH, ionic strength and the isoelectric point of the proteins in concern, is reflected in the zeta-potential analysed.

All protein samples showed positive charges at pH 3, and negative charges at pH 7, which is in line with the literature value of isoelectric point of the patatin protein at ~pH 4.5-5.1 (Barta et al., 2012) and of the protease inhibitors (PI) at ~pH 5.1-9.0 (Povreau et al., 2001). Furthermore, at pH 5 the zeta-potential of the PI fractions was close to 0 mV, while the further fractionation by HIC resulted in the 1st fraction of PI having a higher zeta-potential to the positive side and PI HIC 2 a slightly negative value. For the patatin fraction sample at pH 5, the zeta-potential showed a negative value, meaning that this fraction carried a negative charge, i.e. is above its isoelectric point, which remained in the further fractionated patatin HIC 1 and 2 sample.

**Figure 2.** Zeta-potential (mV) of potato protein samples (spraydried powder, patatin fraction, patatin HIC fraction 1 and 2, PI fraction and PI HIC fraction 1 and 2) at pH values of pH 3, pH 5 and pH 7, n = 4.
All samples at pH 7 were negative, with the powder and patatin fractions resulting in the lowest values. The zeta-potential can also relate to the stability of the proteins in solution, as a numerically higher zeta-potential indicates a higher ability to stay in solution and resist aggregation due to repulsive electrostatic forces. Based on visual turbidity the patatin fraction showed good (solubility/stability) at pH 3 and 7 with a minimum at pH 5, which correlated well with zeta-potential measurements. For the PI fractions, low turbidity was observed at pH 3 with increasing turbidity at pH 5 and pH 7. The PI HIC 1 fraction showed lower turbidity than PI and PI HIC 2 at pH 5, which could be related to the higher zeta-potential of this fraction.

3.3. Foam studies – capacity and stability

The foaming properties of powder, patatin and PI fractions were evaluated by shaken foam capacity (Figure 3) and foam stability as relative volume after 2 hours (Figure 4).

![Figure 3](image-url)

**Figure 3.** Foaming capacity as overrun (OR) of potato protein powder and fractions of c = 1 g/L as function of pH values. Bars are means ± SD, n = 6.
In general, all potato protein fractions and the whole spray dried powder had capacities ranging from ~0.8-1.8 L/L. The patatin HIC fraction 2 (most hydrophobic on basis of elution in HIC) was superior at pH 3 to the other protein samples, while the PI-fractions were much lower at pH 3 compared to the two other samples. At pH 5, less difference between the samples appeared with patatin and PI HIC 2 yielding highest values, whereas at pH 7, the patatin HIC fraction 1 was inferior to all other samples, and here the PI HIC fraction 2 (most hydrophobic PI fraction) revealed the highest foam overrun. To conclude, the patatin fractions showed highest overrun at pH 3, intermediate at pH 5 and lowest at pH 7. The high foam overrun at pH 3 is believed to be related to irreversible denaturation of patatin at low pH (Pots, de Jongh, Gruppen, Hessing, & Voragen, 1998). The intermediate foam volume and pH 5 and low foam volume at pH 7 could be related to the zeta-potential, with pH 5 being closer to pl and pH 7 above. The PI fractions had highest overrun at pH 5, at which the zeta-potential were closest to zero compared to pH 3 and 7, except for the PI HIC 1 fraction at pH 5 that had a higher zeta-potential value (Figure 2). In contrast to our results (van Koningsveld, et al., 2002) found highest foam volume by whipping patatin solutions at pH 5, with a small reduction at pH 3 and a large reduction in foam volume at pH 7. Furthermore the PI fraction showed higher foam volume than patatin at pH 5 and 7, while foam stability was highest for the patatin fractions showing significantly lower liquid drainage at pH 5 and 7 than the PI fraction. These differences may be partly explained by the method of foam productions e.g. has it been shown that sparging results in very low overrun and stability of the PI fraction compared to patatin (Ralet, et al., 2001).
Figure 4. Foam stability 2 hours after foam production as remaining relative foam volume (FV) of potato protein powder and fractions of c = 1 g/L as function of pH values. Bars are means ± SD, n = 6.

Foam stability of whole potato protein powder, the patatin fractions and the PI IEX sample, showed lowest stability at pH 3, this was especially pronounced for the patatin fraction with less than 20 % foam remaining after 2 hours (Figure 4). The two patatin HIC fractions performed different at pH 3, with Patatin HIC 1 having a significantly higher stability compared to Patatin HIC 2 and Patatin. The reason for the stability difference between patatin fractions is not known, but it can be speculated that the presence of phenolic compounds could play a role by altering the hydrophobic character of the proteins (Arntfield, 1996), (Rawel, Kroll, & Rohn, 2001) and that HIC purification decreases the amounts of phenolic substances. The protein fractions of PI HIC 1 and PI HIC 2 showed the highest foam stability among all samples at the pH 3. It has been suggested by (Partzia & Kiosseoglou, 2001) that oxidation of sulphydryl groups of small potato proteins with Mw < 12 kDa play a role in both foam overrun and stability, this observation can however not explain the high stability of the PI fraction since sulphydryl oxidation rate is minimal at pH 3 (Monahan, German, & Kinsella, 1995).
At pH 5 and pH 7, the protein samples had much more comparable foam stability levels. Overall, the PI fraction was least susceptible for the change in pH, and even at pH 5 where the zeta-potential for this fraction was ~0 mV, the foam was stable with 75% foam volume retained 2 hours after foam production. At pH 7, the whole powder and the PI HIC 2 fractions performed highest in foam stability with the overall retention of ~80% foam volume after 2 hours.

Looking closer into the foam decay profiles of the foam volume, these showed clearly a two-step behaviour at pH 3 of the patatin and patatin HIC 2 foam and to a minor degree of the whole powder sample foam, whereas at the other conditions of sample and pH, the foam volume had a one-step decrease profile (Figure 5). The two-step decrease is suggested to be liquid drainage from the foam in the first step followed by foam bubble collapse due to coalescence and Ostwald ripening in the second step. Most likely, this takes place in all the analysed foams as being common destabilising mechanisms of foams (Hammershoj, Prins, & Qvist, 1999), however the change is more drastic at pH 3. Based on Figure 5. Foam volume as function of time after foam production and of pH 3, pH 5 and pH 7 for A) patatin fraction, B) whole protein powder, C) PI fraction, D) Patatin HIC 1 fraction, E) patatin HIC 2 fraction, F) PI HIC 1 fraction, and G) PI HIC2 fraction. Vertical bars indicate +/- standard deviations. Figure 5, the sample with highest foam volume after two hours at pH 3 was patatin HIC 1 (22 ± 1.7 mL) and the second highest was PI HIC 2 (19.7 ± 0.3 mL), at pH 5 the PI HIC 2 performed best (24.4 ± 1.1 mL) and patatin second best (22.8 ± 1.5 mL) and at pH 7, the PI HIC 2 had the highest foam volume (20.6 ± 1.0 mL) and the spray dried powder performed second highest (18.9 ± 0.7 mL).
Figure 5. Foam volume as function of time after foam production and of pH 3, pH 5 and pH 7 for A) patatin fraction, B) whole protein powder, C) PI fraction, D) Patatin HIC 1 fraction, E) patatin HIC 2 fraction, F) PI HIC 1 fraction, and G) PI HIC2 fraction. Vertical bars indicate +/- standard deviations.
3.4. Surface tension

The surface tensions of the whole protein powder at the different pH-values are illustrated in Figure 6 over time until reaching a steady state. For both whole protein powder and the patatin fraction, the final value reached at steady state and the half-life time to reach 50% decrease in surface tension from the initial \( \gamma \) of the various buffers applied are shown in Table 1. The buffers initial \( \gamma \) were in the range of 67.8-71.7 mN/m.

![Surface tension curves](image)

**Figure 6.** Examples of surface tension curves of potato protein powder solutions at pH 3, pH 5 and pH 7 as function of time (s).

For the whole protein powder solution, there was only a small difference in the steady state surface tension as function of pH, with pH 5 conditions resulting in a slightly higher level compared with pH 3 and pH 7. However, the half-life time was much lower at pH 3 in comparison with that at pH 7, which was almost twice as long time to reach the half-life. This could indicate that the potato protein powder solution had highest surface activity at the pH 3, less at pH 5 and lowest at pH 7, which correlates well with the foaming capacity results in Figure 3. Furthermore, the patatin fraction analysed at pH 5 had a much lower half-life time than at pH 7, which again correlates with the foam OR of these two samples (Figure 3).
Table 1 Mean ± S.D. of surface tension as final steady state value and half-life time for 50% decrease for potato protein powder and patatin fraction at c = 0.5 g L\(^{-1}\) as function of pH, n = 3

<table>
<thead>
<tr>
<th></th>
<th>pH 3</th>
<th>pH 5</th>
<th>pH 7</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Whole protein powder</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steady state γ, mN/m</td>
<td>44.01 ± 0.06</td>
<td>43.98 ± 0.39</td>
<td>43.08 ± 0.60</td>
</tr>
<tr>
<td>Half-life time, s</td>
<td>1342 ± 67</td>
<td>1866 ± 161</td>
<td>2722 ± 146</td>
</tr>
<tr>
<td><strong>Patatin fraction</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Steady state γ, mN/m</td>
<td>n.d.</td>
<td>47.50 ± 0.46</td>
<td>42.40 ± 0.49</td>
</tr>
<tr>
<td>Half-life time, s</td>
<td>n.d.</td>
<td>1617 ± 155</td>
<td>2299 ± 172</td>
</tr>
</tbody>
</table>

A relationship between a fast interfacial adsorption and foamability have previously been reported (Marinova, et al., 2009), but here the fastest adsorption was near pI in contrast to our results. Both hydrophobicity and charge play a role in adsorption (Wierenga, et al., 2010), but we suggests that increased hydrophobicity at pH 3 or a structural change is the driving force, overcoming the increase in charge at this pH compared with pH 5.

3.5. Emulsion studies – interfacial tension and emulsification

The interfacial tension of the various protein fractions was analysed at pH 3 (Figure 7). The interfacial tension at equilibrium describes when proteins have adsorbed and rearranged at the oil-water interface (Romero, et al., 2011). When comparing whole protein powder to patatin and PI, did patatin show the lowest value, and PI the highest, with the powder giving an intermediate value. Further fractionation of patatin by HIC resulted in the least hydrophobic fraction (HIC 1), yielding the highest interfacial tension,
while patatin HIC 2 not being significantly different from patatin. Fractionation of the PI fraction lead to the most hydrophobic fraction (HIC 2) yielding a significantly lower value than unfractionated PI.

It has previously been analysed that potato protein isolate reach equilibrium interfacial tensions between 9-12 mN/m after 5 hours dependent on pH and protein concentration (Romero, et al., 2011), which is comparable to our present results (Figure 7).

![Interfacial tension graph](image)

**Figure 7.** Interfacial tension at pH 3 after 9 hours of equilibrium of different fractions of potato proteins, PI = protease inhibitor fraction, HIC = hydrophobic interaction chromatography, n = 3.

Emulsion activity index (EAI) results are shown in Figure 8. The patatin HIC 2 fraction had a significantly higher emulsifying capacity given as interfacial area stabilized per mass of protein, which was ~20% higher than the second-best performing samples, i.e. patatin. The PI fraction showed lower EAI performance. The two HIC fractions of PI performed differently with PI HIC 1 yielding lower values than PI, and PI HIC 2 higher values than PI, where PI HIC 2 was at a level comparable to the patatin fractions.
Furthermore, the emulsion stability index (ESI) being a measure of the diluted emulsion’s stability over time is given in Figure 9. Again the patatin HIC 2 fraction had the highest value, however insignificantly different from the patatin and patatin HIC 1 fraction. PI HIC 1 showed lower stability than PI, and PI HIC 2 higher stability than PI. The PI HIC 2 had values comparable to the patatin fractions.
Figure 10. Mean emulsion droplet diameters given as A) $D_{4.3}$ and B) $D_{3.2}$ for samples of whole potato protein powder, patatin, PI and HIC fractions hereof, $n = 6$.

The emulsion droplet diameters analysed as mean of size distributions is given both as volume weighted (Figure 10A) and as surface weighted (Figure 10B) diameters. Regardless of which diameter the PI fraction produced an emulsion with much larger oil droplets compared with all other samples, and the patatin fraction emulsion appeared to be composed of oil droplets of smaller mean diameter. A significant difference was seen with the HIC fractions of PI, with PI HIC 1 yielding larger, and PI HIC 2 smaller droplets than the PI fraction. This data could partly explain the difference in emulsion activity and stability of the PI fractions, which correlated with oil droplet size. A previous study also found the PI fraction to have the largest oil droplets ($D_{4.3}$) at pH 3, compared to patatin and a total protein isolate, with values of 1.23, 0.84 and 0.73 µm, respectively (van Koningsveld, et al., 2006). In our present experiments, larger droplet sizes were measured than previously reported e.g. 0.58-0.84 µm ($D_{4.3}$) (Romero, et al., 2011) and 0.55-5.79 µm.
(D 4,3) (van Koningsveld, et al., 2006), which can depend on different apparatus being applied in the emulsion preparation. Droplet size have been reported to correlate with patatin amount and more specifically the lipid acyl hydrolase activity, resulting in release of surface active molecules lowering surface tension and giving smaller oil droplets (van Koningsveld, et al., 2006). In our studies, we believe this to be of minor contribution since the enzyme activity should be diminished at pH 3, and furthermore, the use of sunflower oil with the majority of the fatty acids being C18 (Orsavova, Misurcova, Ambrozova, Vicha, & Mlcek, 2015) conflicts with patatin having higher affinity for shorter chain fatty acids (Anderson, Pinsirodom, & Parkin, 2002).

3.6. Emulsion rheological properties

Dynamic rheology of emulsions was performed as frequency sweeps and recordings of storage moduli (G’) are shown in Figure 11. There was a significant difference between the potato protein fractions in their rheological properties, with the patatin HIC 2 fraction having the overall highest G’ values, i.e. the emulsion is thicker in consistency, whereas the PI fraction showed very low G’ values being only one third the level of all other emulsion samples, i.e. this emulsion would appear less thick in consistency. The PI HIC 2 fraction showed a significantly higher G’ than the PI fraction and the response in G’ on frequency was different than the other PI samples. The replicate measurements led to consecutive higher G’ values, indicating that the time length after emulsification could be of importance for the emulsion consistency.
In summary, did the patatin and especially patatin HIC 2 show the best emulsion properties with increased performance compared to the total protein powder. The PI fraction and PI HIC 1 showed worse performance i.e. lower ESI, EAI and larger droplet seize than the whole protein powder. PI HIC 2 did however show a performance equal to the whole powder and patatin fractions. Previous studies at low pH (pH 4) have shown a significant difference between creaming rate of whole protein isolates being ~4 times higher compared to patatin and ~6 times higher compared to the PI fraction, and furthermore showing excessive coalescence of the whole protein isolate emulsions (Ralet, et al., 2000). However, negligible differences in emulsion aggregation at pH 3 between PI, patatin and a total protein fraction are reported (van Koningsveld, et al., 2006).
4. Conclusions

In this study, patatin and protease inhibitor rich fractions obtained by ion exchange chromatography of spray dried potato protein powder, were further purified into low and high hydrophobic fractions based on hydrophobic interaction chromatography, and all samples used for foam and emulsion studies.

Foam overrun for the total protein powder and the all patatin rich fractions were highest at pH 3, with successive lower values at pH 5 and 7. HIC fractionation of patatin led to increased foam overrun and stability when compared to patatin at pH 3.

The protease inhibitor fractions had highest foam overrun at pH 5 with lower values at pH 3 and 7. Highest overrun and stability at pH 5 and 7 was from the most hydrophobic PI fraction.

The spray dried powder and all patatin fractions showed better emulsion stability, smaller oil droplets and higher texture than PI and PI HIC 1, with the best performing fraction of all being patatin HIC 2. Surprisingly the most hydrophobic PI fraction (PI HIC 2) showed emulsion characteristics on par with the spray dried powder and IEX patatin.

This study shows that new and improved functionality can be obtained when potato protein fractions are further purified by hydrophobic interaction chromatography and the generally increased performance was associated with the most hydrophobic fractions of either patatin or protease inhibitor.

Acknowledgements

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References


Paper V

Appearance and textural properties of sheared potato protein isolate gels – impact of drying method, pH and ionic strength

Jesper Malling Schmidt, Henriette Damgaard, Mathias Greve-Poulsen, Lotte Bach Larsen Marianne Hammershøj

Manuscript submitted to LWT – Food Science and Technology
Abstract: The objective was to prepare sheared gels of potato protein concentrate and evaluate the effect of pH (3, ~4, ~7), ionic strength (15 or 200 mM) and protein drying conditions (spray or freeze drying) on the final appearance and rheological characteristics. Heat-set gels (3 g total solids/100 g) at a high ionic strength (200 mM) resulted in an inhomogenous appearance with presence of clots, while low ionic strength (15 mM) gave homogenous structures. Gels prepared at pH 3 became transparent while preparation above pH 3.0 resulted in high turbidity. Heat treatment and cooling resulted in gelation for all samples except freeze dried powder at pH 3.0. Flow curves during shear from 0.1-100 s\(^{-1}\) were fitted by the Herschel-Bulkley model indicating shear thinning behaviour for all samples except the freeze dried sample at pH 3 which displayed a Newtonian behaviour. Oscillatory measurements after shear indicated viscous behaviour (phase angle above 45 °) for the spray dried sample at pH 3, and gelled behaviour (phase angle above 45 °) for the remaining gelled samples. Structure recovery was observed after shear in all samples except at pH 3.0. The data shows potato protein can be used as ingredient in protein beverages.
Veronika Bartova has published Work on potato protein extraction from potato fruit juice.
Submission of original research manuscript to LWT – Food Science and Technology

Dear Editor,

On behalf of the co-authors, I hereby submit the manuscript entitled ‘Appearance and textural properties of sheared low concentration potato protein gels – impact of drying method, pH and ionic strength’.

The manuscript is based on new research on potato proteins and the effect of drying method and different pH conditions for rheological properties of sheared gels relevant for protein beverages. The experimental part has been performed in close collaboration with the potato industry.

We look forward to hear from you.

Kind regards

Marianne Hammershøj
Associate Professor
Corresponding author
Highlights

Sheared gels were made of spray dried or freeze dried potato protein

At ionic strength of 15 mM, sheared gels were homogeneous, but clotted at 200 mM

Gels were transparent at pH 3 and turbid at pH ~4 and pH ~7

Gel strength and flow properties was affected by pH and powder type

Gels were shear thinning, but at pH 3 freeze dried powder gels behaved Newtonian
Appearance and textural properties of sheared low concentration potato protein gels – impact of drying method, pH and ionic strength

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Abstract

The objective was to prepare sheared gels of potato protein concentrate and evaluate the effect of pH (3, ~4, ~7), ionic strength (15 or 200 mM) and protein drying conditions (spray or freeze drying) on the final appearance and rheological characteristics. Heat-set gels (3 g total solids/100 g) at a high ionic strength (200 mM) resulted in an inhomogenous appearance with presence of clots, while low ionic strength (15 mM) gave homogenous structures. Gels prepared at pH 3 became transparent while preparation above pH 3.0 resulted in high turbidity. Heat treatment and cooling resulted in gelation for all samples except freeze dried powder at pH 3.0. Flow curves during shear from 0.1-100 s⁻¹ were fitted by the Herschel-Bulkley model indicating shear thinning behaviour for all samples except the freeze dried sample at pH 3 which displayed a Newtonian behaviour. Oscillatory measurements after shear indicated viscous behaviour (phase angle above 45 °) for the spray dried sample at pH 3, and gelled behaviour (phase angle above 45 °) for the remaining gelled samples. Structure recovery was observed after shear in all samples except at pH 3.0. The data shows potato protein can be used as ingredient in protein beverages.
1. Introduction

It is expected that consumption of protein rich beverages will increase in the coming years with sports nutrition and supplements for elderly as two important growth areas. These beverages are often made with milk proteins or fractions hereof e.g. whey powder which can be formulated at low pH giving a clear and appealing appearance (Villumsen et al., 2015). In recent years many alternative protein sources have emerged from e.g. plants, algae and insects. One such specific plant derived source is potato protein, which is found in potato fruit juice (PFJ) produced as a side stream from the production of potato starch. PFJ contains 2-5 % solids, of which 35 % is N-containing substances, protein, peptides and amino acids (Knorr, Kohler, & Betschart, 1977). The potato proteins have historically been divided into three groups. The first group being the patatins which constitutes ~38-40% of total protein. Patatins are glycoproteins with a mass of 39-43 kDa depending on glycosylation patterns with isoelectric point (pI) in the range of 4.45-5.17 (Baier & Knorr, 2015; Barta, Bartova, Zdrahal, & Sedo, 2012). The second group, the protease inhibitors (PI) represents up to ~50% of total protein (Pouvreau et al., 2001). The mass of of PIs varies from 4.3-20.6 kDa and pI covers a range from
The last group of proteins are mainly composed of oxidative enzymes, like polyphenol oxidase and lipoxygenase and enzymes related to starch synthesis (Jorgensen, Stensballe, & Welinder, 2011).

The potato proteins have a favourable amino acid composition in human nutrition and induce a slow moderate increase in postprandial amino acid levels with no effect on postprandial glucose and insulin and is therefore considered as a good protein source (He, Spelbrink, Witteman, & Giuseppin, 2013).

Only very few studies have so far been published on potato protein gels (Creusot, Wierenga, Laus, Giuseppin, & Gruppen, 2011; Lokra, Helland, Claussen, Straetkvern, & Egeland, 2008) and none of them have studied low protein concentration sheared gels.

The aim of this study was therefore to evaluate appearance and rheological characteristics of a sheared potato protein gel as a simple model for a possible potato protein based beverage.

2. Materials and Methods

2.1. Protein samples

Potato protein isolate powders were provided by KMC, Brande, Denmark, and were manufactured from potato fruit juice from the potato harvest in December 2014. The powders were either spray dried on an Anhydro PSD 55 pilot scale spray dried (SPX FLOW, Soeborg, Denmark) or freeze dried on a lab scale vacuum freeze dryer (ScanVac Coolsafe 55-9, Lynge, Denmark).

2.2. Dry matter determination

Dry matter of the powders was determined by drying ~2 g sample in a HR73 halogen moisture analyzer (Mettler Toledo, Schwerzenbach, Switzerland).

2.3. Sample preparation

The protein powders were suspended as 3 g total solids/100 g by stirring the powders in buffers for one hour at room temperature. The used buffers were 30 mM trisodium citrate dihydrate (pH 3) and 7.5 mM disodium hydrogenphosphate dihydrate (pH 7) all adjusted to an ionic strength of either 15 mM or 200 mM with NaCl.
pH of the potato solutions were analyzed by a PHM 92 pH-meter (Radiometer, Copenhagen, Denmark) and the measured pH is shown in Table 1. Differences were found between the target pH and the actual pH measured, therefore additional solutions were prepared where the protein was suspended in MiliQ water with 15 mM NaCl and adjusted to pH 3.0 by addition of 1 M HCL. In the following, the pH values referred to correspond to the measured value.

The conductivity measured at 25 °C by a CDM 210 Conductivity Meter (Radiometer, Copenhagen, Denmark) is also enclosed in Table 1.

2.4. Dynamic rheological measurements

The protein samples were analysed for rheological properties by transferring 20 ml of sample to a rheometer (AR G2, TA instruments, New Castle, DE, USA) fitted with a cup-and-bob geometry (cup radius 15 mm, bob radius 14 mm) connected to a water-bath ensuring temperature control. To prevent evaporation during heating 1 mL sunflower oil was applied on top of the protein solution. In oscillatory mode, strain (0.01–5%) and frequency (0.01–100 Hz) sweeps were performed to determine the linear viscoelastic region.

The rheological program as see in Fig. 1. The first step was heating from 20 °C to 85°C (5 °C/min), holding at 85 °C for 20 min (step 2), cooling from 85 °C to 5 °C (5 °C/min) (step 3). A measurement was then conducted in oscillatory mode at a frequency of 1 Hz and 0.1% strain with collection of data for storage modulus (G’) and phase angle (step 4). The formed protein gel was subjected to a shear rate sweep (step 5 & 6) ranging from 0.1 to 100 to 0.1 s⁻¹ with measurement of shear stress following a measurement in oscillatory mode with frequency of 1 Hz and strain of 0.1% for 20 min at 5 °C (step 7). A second additional shear sweep (up-down) with the same shear rate as the first cycle was performed (step 8 & 9), followed by a 20 min measurement in oscillatory mode (step 10).

The flow curves were fitted to the Herschel-Bulkley model (equation 1) (Kristo, Miao, & Corredig, 2011) where σ is shear stress (Pas), K the consistency coefficient (Pas⁰), η the flow behavior index (-) and σ₀ the
yield stress (Pas), using Rheology Advantage Data Analysis software version 5.7. Each sample was tested at least in duplicate.

\[ \sigma = K(\dot{\gamma})^n + \sigma_0 \]  

2.5. Appearance of gels after shear

Solutions were prepared as described previously. A 25 mL solution was heated in a 50 mL Falcon tube in a water-bath at 85 °C for 20 min, following cooling in water and storage at 5 °C over night. The gels were sheared by hand with a metal spatula doing 10 revolutions for 30 seconds before being poured into a 60 mm diameter transparent petri dish. Pictures were taken with a digital camera.

2.6. Statistical analysis

The results are presented as means with standard deviations. Significant differences between sample treatments were determined by two-way ANOVA analysis with parameters pH, drying conditions and interactions hereof using GraphPad Prism 6 (GraphPad Software Inc, La Jolla, USA, version 6.01). Differences were regarded to be significant at minimum 95 %-level (P < 0.05).

3. Results and Discussion

3.1. Appearance of gels after shear

The gels were sheared after gelation and cooling, with pH 3.0 I: 15 mM resulting in transparent solutions, “pH 3.7-3.9” and “pH 6.8-6.9” at I: 15 mM showing homogeneous and turbid solutions while “pH 3.7-3.9” and “pH 6.8-6.9” samples at I: 200 mM resulted in highly inhomogeneous solutions with appearance of clots/clumps (Fig. 2). Differences were also seen depending on drying method, with freeze dried protein at pH 3.7 at I: 15 mM yielding more homogenous sheared gels than spray dried protein at pH 3.9 I: 15 mM (Fig. 2).

It should be noted that processing at “pH 3.7-3.9” resulted in a more white appearance than “pH 6.8-6.9”.

Fig. 2
3.2. Rheology of gels

Samples showing a distinct heterogeneous appearance after shearing was not included in the rheological tests i.e. samples with ionic strength of 200 mM were excluded (see Fig. 2B as example). Fig. 3A depicts the gelation process during heating of the protein solutions. From this figure it is possible to determine the gel point, defined as the point in which a rapid increase in G′ is observed. The gel point depended on both mode of drying and pH, with the spray dried sample at pH 3.9 having the lowest gel point at T ~57-58 °C followed by the freeze dried sample at pH 3.7 gel point at T ~ 63-64 °C, with both freeze-and spray dried samples at “pH 6.8-6.9” having similar and higher gel points at T ~ 75-77 °C. The samples adjusted to a pH of 3.0 did not form a gel and G′ did not change during the heating phase, the spray dried sample did however form a weak gel upon cooling (Fig. 1, step 3). The spray dried sample at pH 3.0 had an slightly higher ionic strength than the freeze dried sample (Table 1). Adjustment of the ionic strength in the freeze dried sample did however not induce gel formation. The difference between spray and freeze dried samples is believed to be caused by minor denaturation during spray drying, promoting a more unfolded protein structure where hydrophobic patches may be more exposed on the surface and thereby more readily form aggregates. The absence of a gel point during heating of the samples at pH 3.0 corresponds to repulsive forces dominating at this pH, below pI of both patatin and the protease inhibitors, while “pH 3.7-3.9” and “pH 6.8-6.9” are close to the pI of patatin and the protease inhibitors, respectively. Fig. 3B depicts recovery in structure after the first and second shear cycle during the 20 min oscillation measurements, with a fast recovery during the first few minutes followed by a slower phase. Highest recovery is seen for the two samples at “pH 6.8-6.9” and the freeze dried sample at pH 3.7. Similar structure recovery have been reported for systems like stirred yoghurts (Kristo et al., 2011).

In Table 2, the results of the oscillatory measurements are summarised with recording of storage modulus (G′) and phase angle of the gels before and after the gels had undergone two shearing cycles. Before shearing, the two samples at pH 3.0 show significantly less structure than all other samples with the freeze...
dried sample not forming a gel and the spray dried sample producing a very weak gel. The spray dried sample at pH 3.9 showed significantly weaker structure than the freeze dried sample at pH 3.7 when comparing the storage modulus whereas no difference was seen between the drying methods at pH 6.8-6.9.

The two shearing cycles resulted in a sequential decrease in $G'$ and an increase in phase angle, indicating that the initial structure was broken down and that the sample properties had changed towards more viscous behaviour and less elastic behaviour. The spray dried sample at pH 3.0 was having a more viscous structure (phase angle $> 45^\circ$) and the samples at higher pH showing a more elastic gel-structure (phase angle $< 45^\circ$).

Both $G'$ and phase angle were significantly affected by pH, drying method and interactions hereof, except for the phase angle after the second shear cycle, where the effect of drying method was not significant.

**Table 2**

In Fig. 4A, the upward and downward flow curves are depicted for the second shearing cycle of the protein gels. The curves were fitted to the Herschel-Bulkley model and the resulting constants summarized in Table 3. The $K$ (consistency coefficient) was affected significantly by drying method, pH and interactions hereof at all instances ($P < 0.05$) while $\eta$ (flow behavior index) was significantly affected by pH and the interaction between pH and drying method for the first shear cycle ($P < 0.05$), by pH, drying method and the interaction hereof in second shear cycle (up and down).

In all three model fits (Table 3), the spray dried sample pH 6.9 had the highest $K$ and lowest $\eta$ indicating a shear thinning behavior, while the samples at “pH 3.7-3.9” had lower $K$ values and a $\eta$ close to 1 indicating a more Newtonian behavior (Steffe, 1996). The spray dried sample at pH 3.0 showed a shear thinning behavior, while the freeze dried sample showed a pure Newtonian response (Fig. 4B). In Newtonian fluids are shear stress directly proportional to the shear rate, while the stress for shear thinning fluids level off as shear increases, because molecules orient their major axes in the direction of the applied flow and/or weakly associated structures dissociate from each other (Damodaran, 1996).

A hysteresis loop between the up and down shear rate sweep was found, which was more pronounced for the “pH 6.8-6.9 samples”
No clear correlations were found between measurements conducted in oscillation mode (Table 2) and flow mode (Table 3). This indicates that the protein samples and the protein aggregates present herein behave differently while at rest or when under deformation. An explanation for the observed differences may be related to the type of protein network formed. The protein networks formed at pH 3 are proposed to be of the fine-stranded type producing transparent gels at pH values far from pI, while the gels formed at pH 3.7-3.9 and 6.8-6.9 are near pI and form particulate type gels (Stading & Hermansson, 1990). It is proposed that the aggregates formed at pH 3.7-3.9 are denser compared to pH 6.8-6.9 and hereby absorbing more light, producing a gel of whiter appearance, which is in accordance with the visual appearance illustrated in Fig. 2. A denser structure of the aggregates could also explain the lower shear stress of the pH 3.7-3.9 samples, when compared to the other samples due to a smaller hydrodynamic volume. The pH of 3.7-3.9 is also close to the solubility minimum for potato proteins (van Koningsveld et al., 2001), thus resulting in a lower “effective” protein concentration that can participate in gel formation.

Further studies are needed to explain the observed differences, with knowledge of the aggregate size being of prime importance. Protein enriched beverages are typically processed at high temperatures of 88-120 °C for 20-180 s (Villumsen et al., 2015), which differ considerably from the conditions of the present study. Hence, future studies should therefore mimic such process conditions in order to reveal the suitability of potato proteins for protein enriched beverage applications.

4. Conclusions

Spray and freeze dried potato protein powder were used for production of 3 g/100 g sheared gels at pH 3; 3.7-3.9 and 6.8-6.9 with ionic strength of 15 or 200 mM. High ionic strength resulted in a heterogenous gel-structure after shearing, while a low ionic strength gave homogenous structures. The visual appearance of the
gels was affected by pH, with pH 3.0 yielding a transparent solution while pH 3.7-3.9 and 6.8-6.9 had a turbid appearance. Heat treatment and cooling resulted in gelation for all samples except for freeze dried powder at pH 3.0. Based on oscillatory measurements the spray dried sample at pH 3.0 behaved as a viscus fluid after shear with a phase angle above 45°, while the remaining samples had the response of a gel. No apparent correlation was found between the storage modulus and phase angle and the rheological constants $K$ (consistency coefficient) and $\eta$ (flow behavior index) from flow curve fits by the Herschel-Bulkley model. The freeze dried sample at pH 3.0 gave a pure Newtonian response to an increase in shear rate, while the remaining samples displayed a shear thinning and thixotropic response, with samples at pH 6.8-6.9 showing the highest degree of shear thinning. This study shows that there is potential for potato protein to be used as a functional ingredient in sheared protein beverages at specific salt and pH conditions.

Acknowledgments

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References


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**Table 1**

Measured pH and conductivity of the different protein solutions.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Used buffer</th>
<th>Measured pH</th>
<th>Conductivity (mS/cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spray dried</td>
<td>Adjusted with HCL</td>
<td>3.0</td>
<td>4.7</td>
</tr>
<tr>
<td>Freeze dried</td>
<td>Adjusted with HCL</td>
<td>3.0</td>
<td>3.7</td>
</tr>
<tr>
<td>Spray dried</td>
<td>pH 3, I: 15 mM</td>
<td>3.9</td>
<td>2.3</td>
</tr>
<tr>
<td>Freeze dried</td>
<td>pH 3, I: 15 mM</td>
<td>3.7</td>
<td>1.8</td>
</tr>
<tr>
<td>Spray dried</td>
<td>pH 7, I: 15 mM</td>
<td>6.9</td>
<td>2.6</td>
</tr>
<tr>
<td>Freeze dried</td>
<td>pH 7, I: 15 mM</td>
<td>6.8</td>
<td>2.0</td>
</tr>
</tbody>
</table>
Table 2

Rheological characteristics of gels measured in oscillation mode. The storage modulus ($G'$) and phase angle was measured at 5 °C after completion of the temperature program. Measurements were conducted before shearing and after the two shear cycles up to $\gamma = 100 \text{ s}^{-1}$. Step no. refers to Fig. 1.

<table>
<thead>
<tr>
<th>Oscillation mode</th>
<th>Step 4 - Gel</th>
<th>Step 7 - After first shear cycle</th>
<th>Step 10 - After second shear cycle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$G'$ (Pa)</td>
<td>Phase angle (°)</td>
<td>$G'$ (Pa)</td>
</tr>
<tr>
<td>Spray pH 3.0</td>
<td>1.5 ± 0.3$^A$</td>
<td>40.4 ± 2.8$^A$</td>
<td>0.16 ± 0.05$^A$</td>
</tr>
<tr>
<td>Freeze pH 3.0</td>
<td>&lt;d.l.$^X$</td>
<td>&lt;d.l.</td>
<td>&lt;d.l.</td>
</tr>
<tr>
<td>Spray pH 3.9</td>
<td>216.9 ± 20.6$^B$</td>
<td>10.8 ± 0.1$^B$</td>
<td>5.4 ± 1.4$^A$</td>
</tr>
<tr>
<td>Freeze pH 3.7</td>
<td>625.0 ± 22.7$^C$</td>
<td>10.5 ± 0.1$^B$</td>
<td>23.7 ± 3.4$^B$</td>
</tr>
<tr>
<td>Spray pH 6.9</td>
<td>629.3 ± 25.7$^C$</td>
<td>8.9 ± 0.1$^B$</td>
<td>28.6 ± 5.1$^B$</td>
</tr>
<tr>
<td>Freeze pH 6.8</td>
<td>628.8 ± 14.1$^C$</td>
<td>8.9 ± 0.0$^B$</td>
<td>27.7 ± 5.7$^B$</td>
</tr>
</tbody>
</table>

$^X$ <d.l. below detection limit.

Each data point represents the mean ± SD of minimum two replicates.

$^A$-$^D$ Different superscript letters in the same column indicate significant differences at $P < 0.05$. 
Table 3

Rheological constants, $K$ (consistency coefficient), $\eta$ (flow behavior index) of flow curves during shear from 0.1-100 s$^{-1}$ (upward) or 100-0.1 s$^{-1}$ (downward) using the Herschel-Bulkley model. Step no. refers to Fig. 1.

<table>
<thead>
<tr>
<th>Flow mode</th>
<th>Step 6 - First shear cycle (downward)</th>
<th>Step 8 - Second shear cycle (upward)</th>
<th>Step 9 - Second shear cycle (downward)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K$ (Pas$^s$)</td>
<td>$\eta$ (-)</td>
<td>$K$ (Pas$^s$)</td>
</tr>
<tr>
<td>Spray pH 3.0</td>
<td>0.11 ± 0.02$^A$</td>
<td>0.82 ± 0.02$^A$</td>
<td>0.15 ± 0.02$^A$</td>
</tr>
<tr>
<td>Freeze pH 3.0</td>
<td>0.002 ± 0.00$^A$</td>
<td>1.04 ± 0.01$^B$</td>
<td>0.004 ± 0.0$^A$</td>
</tr>
<tr>
<td>Spray pH 3.9</td>
<td>0.03 ± 0.01$^A$</td>
<td>0.97 ± 0.07$^B$</td>
<td>0.05 ± 0.01$^A$</td>
</tr>
<tr>
<td>Freeze pH 3.7</td>
<td>0.07 ± 0.02$^A$</td>
<td>0.84 ± 0.06$^A$</td>
<td>0.14 ± 0.05$^A$</td>
</tr>
<tr>
<td>Spray pH 6.9</td>
<td>0.98 ± 0.07$^B$</td>
<td>0.51 ± 0.03$^C$</td>
<td>2.81 ± 0.22$^B$</td>
</tr>
<tr>
<td>Freeze pH 6.7</td>
<td>0.68 ± 0.13$^C$</td>
<td>0.58 ± 0.04$^C$</td>
<td>1.17 ± 0.31$^C$</td>
</tr>
</tbody>
</table>

Each data point represents the mean ± SD of minimum two replicates.

$A-F$ Different superscript letters within a column indicate significant different samples ($P < 0.05$).
Fig. 1. Schematic representation of the ten steps in the rheological program displaying development in texture during the various phases as well as the use of either oscillatory or flow mode measurements.

Fig. 2. Pictures of gels after heating, cooling and shearing as function of drying conditions, pH and ionic strength (I). Spray dried pH 6.9 I: 15 mM (A), spray dried pH 6.9 I: 200 mM (B), freeze dried pH 3.7 I: 15 mM (C), spray dried pH 3.9 I: 15 mM (D), freeze dried pH 3.0 I: 15 mM with squared paper underneath (E) to illustrate the transparency / turbidity. The diameter of the pictures corresponds to 6 cm.

Fig. 3. Example of gelation profile (step 1 in Fig. 1) of protein solutions during heating (A). Example of oscillatory measurements (step 7 and 10 in Fig. 1) measured at 5 °C after the first and second shear cycles (marked by arrows), the time is shown as total elapsed time from the beginning of the thermal program (B), (●) spray dried pH 3.0; (○) freeze dried pH 3.0; (▼) spray dried pH 3.9; (△) freeze dried pH 3.7; (■) spray dried pH 6.9; (□) freeze dried pH 6.8.
Fig. 4. Example of flow curves of the second shear cycle (step 8 and 9 in Fig. 1) of gels made of spray dried or freeze dried protein powder (A). Example of flow curve of freeze dried protein at pH 3.0 during the second shear cycle (step 8 in Figure 1) (B). (●) spray dried pH 3.0; (○) freeze dried pH 3.0; (▼) spray dried pH 3.9; (△) freeze dried pH 3.7; (■) spray dried pH 6.9; (□) freeze dried pH 6.8.
Figure 1

The graph illustrates the changes in relative texture/stress during different stages:

1. Heating 20 - 85°C
2. Holding 85°C
3. Cooling 85 - 5°C
4. Holding 5°C
5. Shear 1 0.1-100 s⁻¹ (Up)
6. Shear 1 100-0.1 s⁻¹ (Down)
7. Holding 5°C
8. Shear 2 0.1-100 s⁻¹ (Up)
9. Shear 2 100-0.1 s⁻¹ (Down)
10. Holding 5°C
Figure 2
Figure 3

A) 

B)
Figure 4

A)  

B)
Patent application: A method for providing several fractions of patatin and protease inhibitors

Inventors: Jesper Malling Schmidt, Marianne Hammershøj, Lotte Bach Larsen
A METHOD FOR PROVIDING SEVERAL FRACTIONS OF PATATINS AND PROTEASE INHIBITORS

5 Technical field of the invention

The present invention relates to a method for fractionating patatin and protease inhibitor from a fruit juice. In particular the present invention relates to a method of first fractionating potato fruit juice (PFJ) into an initial patatin isolate and an initial protease inhibitor isolate, followed by separating the initial patatin isolate into several isolates of patatin and separating the initial protease inhibitor isolate into several isolates of protease inhibitors.

Background of the invention

15 Over the years a lot of research has been invested in the field of fractionating complex protein containing mixtures and complex protein mixtures obtained from vegetables are no exception. In particular, fractionation of potatoes, such as potatoes belonging to the Solanaceae or nightshade family is of particular interest. For potatoes, in particular the swollen portion of the underground stem, called a tuber, is designed to provide food for the green leafy portion of the plant, seem to be an interesting source for protein fractionation providing isolates with different functionalities.

The most relevant fraction of e.g. potatoes, for fractionation may be the fruit juice portion of the potato tubers. Potato fruit juice may be obtained from the production of potato starch and may have been considered to provide environmental problems when being disposed. Hence, instead of disposing the potato fruit juice, one possibility may be to recover the functional proteins.

Fresh potato juice is a complex mixture of soluble and insoluble material comprising proteins, starch, minerals, toxic glycoalkaloides, and reactive phenols. The oxidation of natural phenolic compounds in potato juice causes them to turn brown or black. The phenolic compounds may be oxidized into quinones, which combine easily into a dark polymer residue. During this oxidation process reaction and partial cross-linking of the proteins may occur very rapidly. Thus, the complexity and instability of fruit juice, in particular potato juice, makes the separation and isolation of minimally denatured or modified potato proteins, hence native proteins, very complicated, challenging and
economically demanding compared to the isolation of proteins from other types of protein sources.

Potato fruit juice comprises two major groups of proteins, (i) the patatin family, being 39-43 kDa (kilo Dalton) glycoproteins having a glycosylation of 1-3 per protein and being present in an amount of up to 40% of the total amount of protein present in the potato fruit juice, and (ii) protease inhibitors, being 4.3-20.6 kDa proteins and may often be classified in 7 sub-groups and being present in an amount of up to 50% of the total amount of protein present in the potato fruit juice.

Document WO 2014/011042 relates to a method of obtaining two protein isolates from potato juice using a specific absorbent. The method comprises adjusting the pH of the potato fruit juice to 4.0-6.5 and load the potato fruit juice to the absorbent material having at least 90% of pores with a diameter between 10 and 200 nm. Further, the adsorbent material is functionalized with a hydrophobic mixed mode ligand. The potato protein is desorbed from the carrier by elution and the protein is optionally further concentrated and/or dried.

WO 2008/092450 relates to large-scale fractionation and isolation of proteins from potatoes, using an adsorbent material coupled with a ligand for capturing of proteins. Hereby a method for fractionating patatin and protease inhibitors may be provided. The adsorbent used in WO 2008/092450 is a functionalized matrix polymer carrying a plurality of covalently attached functional groups comprising an aromatic or heteroaromatic ring-system and one or more acidic groups.

US 2010/0040591 describes a process for obtaining a native potato protein isolate comprising patatin and protease inhibitor, comprising:

- subjecting potato fruit juice to a flocculation by a divalent metal cation at a pH of 7-9;
- centrifuging the flocculated potato fruit juice, thereby forming a supernatant;
- subjecting the supernatant to expanded bed adsorption chromatography operated at a pH of less than 11 and a temperature of 5-35° C. using an adsorbent capable of binding potato protein, thereby adsorbing the native potato protein to the adsorbent; and
- eluting at least one native potato protein isolate from the adsorbent with an eluent.
Despite the technology described in the prior art, there is an continuing interest and a need in the industry for a new method for providing patatin isolates and protease inhibitor isolates that maintains the native potato proteins from the fruit juice and maintain the functional properties of the various proteins, providing isolates having strong and specific functional properties, such as emulsifying capacity, foaming capacity, thermo-gelling capacity, water binding capacity, and water solubility.

Hence, a method, which is efficient, reliable, simple and suitable for industrial scale application, for providing several isolates (rich in patatins and rich in protease inhibitors) comprising native and stabile proteins from fruit juice would be advantageous.

**Summary of the invention**

Thus, an object of the present invention relates to a method for fractionating the proteins patatin and protease inhibitor from a fruit juice by an initial fractionation of patatin and protease inhibitor followed by a further fractionation of the initial patatin isolate into two or more patatin isolates and a further fractionation of the initial protease inhibitor isolate into two or more protease inhibitor isolates and whereby the protein isolates comprise native, or substantially native, proteins.

In particular, it is an object of the present invention to provide a method that solves the above mentioned problems of the prior art with functional properties, efficiency, reliability, and applicable for industrial scale.

Thus, one aspect of the invention relates to a method for obtaining at least two patatin isolates and at least two protease inhibitor isolates, said method comprises the steps of:

(i) providing fruit juice;

(ii) subjecting the fruit juice to an initial fractionation process obtaining an initial patatin isolate and an initial protease inhibitor isolate;

(iii) subjecting the initial patatin isolate to a chromatographic separation material providing a first patatin isolate and a second patatin isolate;
subjecting the initial protease inhibitor isolate to a chromatographic separation material providing a first protease inhibitor isolate and a second protease inhibitor isolate;

wherein the chromatographic separation material is selected from a hydrophobic material, for performing hydrophobic interaction chromatography (HIC), or a reversed phase, for performing reversed phase chromatography (RPC), preferably, the chromatographic separation material is a hydrophobic material.

Another aspect of the present invention relates to a patatin isolate obtainable by the method according to the present invention.

Yet another aspect of the present invention relates to a protease inhibitor isolate obtainable by the method according to the present invention.

Still another aspect of the present invention relates to a food product comprising the patatin isolate and/or a protease inhibitor isolate according to the present invention.

A further aspect of the present invention relates to the use of a patatin isolate and/or a protease inhibitor isolate according to the present invention, in a food product or a feed product or as a foaming agent, a gelling agent, or an emulsifying agent.

Brief description of the figures

Figure 1 shows a schematic flowchart of the purification process with either potato fruit juice (PFJ) directly provided or potato fruit juice (PFJ) dissolved potato protein as starting material.

The present invention will now be described in more detail in the following.

Detailed description of the invention

Fruit juice, such as potato fruit juice is considered a by-product of the starch industry, and contains proteins with interesting functionalities, such as protease inhibitors or patatin with high nutritional values. Due to their functional properties and their content in potato tubers, these proteins are principally of industrial interest. A drawback for the application of these potato proteins is the fractionation and isolation of the proteins under
maintenance of the biological activity. The methods described so far in the literature, does not balance the requirements between costs and the fractionation performance in a financial attractive manner.

The present invention describes a chromatographic approach using sequential fractionating methods for fractionating fruit juice into separate protein fractions, in particular into separate patatin isolates and protease inhibitor isolates. Additionally, the content of toxic glycoalkaloids, polyphenol oxidase (PPO), reactive phenols and dark colouring agents naturally occurring in potatoes may be significantly reduced during the preparation of the different patatin isolates and the various protease inhibitor isolates.

Hence, the present invention relates to a method for obtaining at least two patatin isolates and at least two protease inhibitor isolates, said method comprises the steps of:

(i) providing fruit juice;

(ii) subjecting the fruit juice to an initial fractionation process obtaining an initial patatin isolate and an initial protease inhibitor isolate;

(iii) subjecting the initial patatin isolate to a chromatographic separation material providing a first patatin isolate and a second patatin isolate;

(iv) subjecting the initial protease inhibitor isolate to a chromatographic separation material providing a first protease inhibitor isolate and a second protease inhibitor isolate;

wherein the chromatographic separation material is selected from a hydrophobic material, for performing hydrophobic interaction chromatography (HIC), or a reversed phase, for performing reversed phase chromatography (RPC), preferably, the chromatographic separation material is a hydrophobic material.

In the context of the present invention, the term "at least two patatin isolates" relates to at least two different isoforms of patatin from the patatin family comprising 39-43 kDa glycoproteins having a glycosylation of 1-3 glycosidic moieties per protein. In an embodiment of the present invention the fractionation results in at least 3 patatin isolates, such as at least 4 patatin isolates, e.g. at least 5 patatin isolates.

In the context of the present invention, the term "at least two protease inhibitor isolates" relates to at least two different fraction comprising different forms of protease inhibitors,
comprising proteins having a molecular size between 4.3-20.6 kDa. Preferably, the different forms of protease inhibitors may be classified in different sub-groups. In an embodiment of the present invention the fractionation results in at least 3 protease inhibitors isolates, such as at least 4 protease inhibitors isolates, e.g. at least 5 protease inhibitors isolates.

The method according to the present invention starts by providing a fruit juice. Said fruit juice may be provided directly from, e.g. a starch factory as a side stream or it may be provided as a dissolved fruit juice obtained from either dried fruit juice or dried fruit juice proteins.

In the event the fruit juice is provided from dried fruit juice or dried fruit juice proteins the powder may be dissolved in a buffer and adjusted to the desired pH value and the desired conductivity and stirred for 1-90 minutes, such as 20-75 minutes, e.g. 30-60 minutes followed by e.g. centrifugation at 4000 rpm 15 min.

In an embodiment of the present invention, the fruit juice is obtained from potato, preferably potato tubers, even more preferably, the fruit juice is obtained from tubers from the Solanaceae or nightshade family, in particular Solanum tuberosum.

In order to prepare the fruit juice for the fractionation method according to the present invention pH and/or conductivity of the fruit juice may be adjusted. In an embodiment of the present invention the pH of the fruit juice may be adjusted to a pH in the range of 7-10, such as in the range of 7.5-9.0, e.g. in the range of 7.7-8.5, such as about pH 8.0.

In a further embodiment of the present invention the fruit juice may be adjusted to a conductivity of less than 50 mS/cm, such as less than 40 mS/cm, e.g. less than 30 mS/cm, such as less than 25 mS/cm, e.g. less than 20 mS/cm, such as less than 15 mS/cm.

During the pH adjustment and/or the adjustment of the conductivity fibres, proteins, and other materials may precipitate from the solution and these precipitates may preferably be removed before the initial fractionation process in order not to disturb or influence on the initial fractionation process. Hence, in an embodiment of the present invention the fruit juice may be subjected to centrifugation or filtration before the initial fractionation of the fruit juice in step (ii).

The initial fractionation process is provided in order to separate the patatin molecules from the protease inhibitor molecules. Several fractionation processes are available for
separating patatin from protease inhibitor. In an embodiment of the present invention the initial fractionation process may be a chromatographic separation process, a filtration process, a centrifugation process, precipitation process or a combination hereof.

In an embodiment of the present invention the chromatographic separation process may be selected from the group consisting of an ion exchange chromatographic process, a mixed mode chromatographic process, an affinity chromatographic process or a combination hereof, preferably the chromatographic separation process is an ion exchange process.

Preferably, the ion exchange chromatographic process may be an anion exchange chromatographic process.

In order to facilitating the chromatographic separation process the chromatographic separation process may involve a chromatographic separation material. In a preferred embodiment of the present invention the initial patatin isolate may be retained during the initial fractionation process and/or wherein the initial protease inhibitor isolate is found in the run through fraction obtained from the initial fractionation process.

After having a protein, such as the patatin, retained in the initial fractionation process the initial patatin isolates retained may be eluted by addition of a first elution buffer.

In an embodiment of the present invention the first elution buffer comprises 0.5-2M salt, such as 0.75-1.5M salt, e.g. about 1M salt and/or the elution buffer has a pH in the range of 7-10, such as in the range of 7.5-9.0, e.g. in the range of 7.7-8.5, such as about pH 8.0.

Adjustment of the salt content of the first elution buffer may be done by addition of a salt, preferably the salt is NaCl.

In order to optimise productivity of the elution process different modes of elution may be applied. In an embodiment of the present invention the elution may be performed in a single step elution, in a two step elution, in a multi-step elution or as a gradient. Preferably the elution may be performed in a two step elution method.

The initial paratin isolate and/or the initial protease inhibitor isolate obtained may be further fractionated providing two or more patatin isolates and two or more protease inhibitor isolates. In an embodiment of the present invention the initial patatin isolate
and/or the initial protease inhibitor isolate may be concentrated, preferably by ultra-filtration, nano-filtration, micro-filtration and/or dia-filtration.

Before the initial patatin isolate and/or the initial protease inhibitor isolate are subjected to the chromatographic separation material the conductivity of the initial patatin isolate may be adjusted to a conductivity in the range of 60-170 mS/cm, such as 100-130 mS/cm and the conductivity of the initial protease isolate may be adjusted to a conductivity in the range of 60-170 mS/cm, such as 110-140 mS/cm.

In order to optimise the separation process when contacting the initial patatin isolate and/or the initial protease inhibitor isolate with the chromatographic separation material, the initial patatin isolate and/or the initial protease isolate may be subjected to a concentration treatment prior to the conductivity adjustment. Preferably, the concentration treatment comprises ultra-filtration, nano-filtration and/or micro-filtration.

In a preferred embodiment of the present invention, the chromatographic separation material may be a hydrophobic material, for performing hydrophobic interaction chromatography (HIC), or a reversed phase, for performing reversed phase chromatography (RPC). Preferably, the chromatographic separation material may be a hydrophobic material.

In the present context, the term “chromatographic separation material” relates to a material in the form of a stationary phase used in a process for separating components (patatin and protease inhibitor) present in a mobile phase, e.g. the initial patatin isolate and the initial protease inhibitor isolate, resulting in the stationary phase withhold one or more components from the mobile phase.

In an embodiment of the present invention the hydrophobic material may comprise a ligand selected from the group consisting of alkyl-groups or aryl-groups. In particular, the hydrophobic material may comprise a ligand selected from the group consisting of butyl-groups; octyl-groups and phenyl-groups.

The various fractions of patatin isolates and/or protease inhibitor isolates may be obtained by subjecting the chromatographic separation material to a second elution buffer. Depending on the conductivity and choice of gradient of the second elution buffer, 1 protease inhibitor isolate can be collected at high conductivity (> 100 mS/cm), 1 protease inhibitor isolate can be collected at medium conductivity (100-20 mS/cm) and 1 protease inhibitor isolate can be collected at low conductivity (< 20 mS/cm). 1 patatin isolate can
be collected at medium conductivity (100-20 mS/cm) and 1 patatin isolates can be collected at low conductivity (< 20 mS/cm).

In an embodiment of the present invention the at least two patatin isolates and/or the at least two protease inhibitor isolates are eluted from the chromatographic separation material by the addition of a second elution buffer.

In a preferred embodiment of the present invention the at least two patatin isolates and/or the at least two protease inhibitor isolates are eluted from the chromatographic separation material by using a stepwise elution wherein the salt content is changing from a high salt content to a low salt content during the elution.

In the present context, the term "high salt content" relates to a salt content in the range of 0.5-3M salt, such as in the range of 1-2M, e.g. in the range of 1.25-1.75M. In the present context, the term "low salt content" relates to a salt content below 0.5M, such as below 0.3M, e.g. below 0.1M, such as below 0.05M, e.g. below 0.01M, such as below 0.005M.

In an embodiment of the present invention the second elution buffer has a pH value in the range of 5.0-10, such as in the range of 5.5-9.5, e.g. in the range of 6.0-9.0, such as in the range of 7.0-8.5, e.g. about pH 8.0.

The at least two patatin isolates and the at least two protease inhibitor isolates may be concentrated, e.g. by using ultra-filtration, dia-filtration, nano-filtration, and/or micro-filtration in order to improve transport costs or to prepare the isolates for drying.

Preferably, the at least two patatin isolates and/or the at least two protease inhibitor isolates may be dried by spray drying, freeze drying, or vacuum drying, preferably by spray drying.

As mentioned above an aspect of the present invention relates to two or more patatin isolates obtainable by the method according to the present invention. Preferably, the patatin isolate according to the present invention comprises substantially native proteins.

As mentioned above an aspect of the present invention relates to two or more protease inhibitor isolates obtainable by the method according to the present invention. Preferably, the protease inhibitor isolate according to the present invention comprises substantially native proteins.
The patatin isolates and/or the protease inhibitor isolates obtainable by the present invention may form part of a food product, a feed product or a beverage product. Preferably, one or more patatin isolates and/or protease inhibitor isolates are suitable as a foaming agent, as a gelling agent, or as an emulsifying agent.

Figure 1 shows the method for fractionating patatin and protease inhibitor from fruit juice, such as potato fruit juice, as described by the present invention.

Initially, the fruit juice, e.g. the potato fruit juice, is provided either as an aqueous solution or as a powder of dried fruit juice or dried fruit juice protein. In the event a powder is used the dried fruit juice or dried fruit juice protein is dissolved in an aqueous media, and stirred for 1-90 minutes, such as 20-75 minutes, e.g. 30-60 minutes to facilitate solubility of the powder in the aqueous media. After dissolving the powder some un-dissolved matter may still be present in the dissolved PFJ which may be removed by centrifugation e.g. at 4000 rpm for 15 min, or by filtration.

The PFJ or the dissolved PFJ may be pH adjusted and/or have the conductivity adjusted before being subjected to an initial fractionating process. Preferably, the PFJ or the dissolved PFJ may be adjusted to a pH value of pH in the range of 7-10, such as in the range of 7.5-9.0, e.g. in the range of 7.7-8.5, such as about pH 8.0 and/or the conductivity of the PFJ or the dissolved PFJ may be adjusted to less than 50 mS/cm, such as less than 40 mS/cm, e.g. less than 30 mS/cm, such as less than 25 mS/cm, e.g. less than 20 mS/cm, such as less than 15 mS/cm.

The PFJ or the dissolved PFJ may subsequently be subjected to an initial fractionation process, such as a chromatographic separation process, a filtration process, a centrifugation process, precipitation process or a combination hereof, preferably the initial fractionation process is a chromatographic separation process resulting in two fractions a patatin-rich fraction (the initial patatin isolate) and a protease inhibitor rich fraction (the initial protease inhibitor isolate). In particular, the chromatographic separation process may be an ion exchange chromatographic method, IEX. In particular an anion exchange chromatographic method.

The patatin-rich fraction (the initial patatin isolate) and the protease inhibitor rich fraction (the initial protease inhibitor isolate) may then be subjected to a chromatographic separation material providing at least a first patatin isolate and a second patatin isolate and at least a first protease inhibitor isolate and a second protease inhibitor isolate. The chromatographic separation material is selected from a hydrophobic material, for performing hydrophobic interaction chromatography (HIC), or a reversed phase, for
performing reversed phase chromatography (RPC), preferably, the chromatographic separation material is a hydrophobic material. The hydrophobic material may comprise a ligand selected from the group consisting of alkyl-groups or aryl-groups. In particular, the hydrophobic material may comprise a ligand selected from the group consisting of butyl-groups; octyl-groups and phenyl-groups.

The at least first patatin and second patatin isolates and the at least first protease inhibitor and second protease inhibitor isolates may comprise improved specific functional properties, such as emulsifying capacity, foaming capacity, thermo-gelling capacity, water binding capacity, and water solubility. Preferably, the at least first patatin and second patatin isolates and the at least first protease inhibitor and second protease inhibitor isolates may be used in an ingredient, in a food product, in a feed product, or in a beverage.

It should be noted that embodiments and features described in the context of one of the aspects of the present invention also apply to the other aspects of the invention.

The invention will now be described in further details in the following non-limiting examples.

**Examples**

**Example 1**

Potato fruit juice (PFJ) is provided and adjusted to pH 8 with NaOH and centrifuged at 4000 rpm 15 min.

PFJ is loaded on an anion exchange column in an amount of 4-8 column volumes with a flow of 12 ml/min.

Elution is done with 25 mM Tris pH 8 buffer with 1 M NaCl in one step using a 30 % elution buffer having a high salt content.

NaCl is added to the initial protease inhibitor isolate (PI) and the initial patatin isolate to reach a conductivity of 100-130 mS/cm for the initial patatin isolate and 110-140 mS/cm for the initial protease inhibitor isolate.

Both fractions (the initial protease inhibitor isolate (PI) and the initial patatin isolate) are centrifuged at 4000 rpm 15 min and the isolates are loaded onto two different hydrophobic
materials substituted with butyl groups. A content of 1-4 column volumes are loaded at a flow rate of 1.2 ml/min.

The bound proteins (the protease inhibitor isolates (PI) and the patatin isolates) are eluted stepwise from 100 % high salt pH 8 buffer to pure water. 3 fractions comprising either protease inhibitor isolates and 2 fractions comprising patatin isolates are collected at medium (100-20 mS/cm) and low conductivity (< 20 mS/cm), respectively, from each of the different hydrophobic materials.

Coloured compounds will elute at high conductivity.

The resulting protease inhibitor isolates (PI) and patatin isolates are concentrated with 3-10 kD (kilo Dalton) molecular weight cut off values using ultra-filtration filters and then subjected to diafiltration with water.

The resulting concentrated protease inhibitor isolates (PI) and concentrated patatin isolates dried, using spray drying.

Example 2

Potato fruit juice (PFJ) is provided and adjusted to pH 8 with NaOH and centrifuged at 4000 rpm 15 min.

PFJ is loaded on an anion exchange column in an amount of 4-8 column volumes with a flow of 12 ml/min.

Elution is done with 25 mM Tris pH 8 buffer with 1 M NaCl in two steps. First using 10 % elution buffer having a high salt content followed by 30 % elution buffer having a high salt content. The two-step elution results in a more pure patatin fraction.

NaCl is added to the initial protease inhibitor isolate (PI) and the initial patatin isolate to reach a conductivity of 80-120 mS/cm for the initial patatin isolate and 80-150 mS/cm for the initial protease inhibitor isolate.

Both fractions (the initial protease inhibitor isolate (PI) and the initial patatin isolate) are centrifuged at 4000 rpm 15 min and the isolates are loaded onto two different hydrophobic materials substituted with butyl groups. A content of 1-4 column volumes are loaded at a flow rate of 1.2 ml/min.
The bound proteins (the protease inhibitor isolates (PI) and the patatin isolates) are eluted stepwise from 100% high salt pH 8 buffer to pure water. 3 fractions comprising either protease inhibitor isolates and 3 fractions comprising patatin isolates are collected at high (> 100 mS/cm), medium (100-20 mS/cm) and low conductivity (< 20 mS/cm), respectively, from each of the different hydrophobic materials.

Coloured compounds will elute at high conductivity.

The resulting protease inhibitor isolates (PI) and patatin isolates are concentrated with 3-10 kD molecular weight cut off values using ultra-filtration filters and then subjected to dia-filtration with water.

The resulting concentrated protease inhibitor isolates (PI) and concentrated patatin isolates dried, using spray drying.

Example 3

Spray dried potato protein comprising patatin and protease inhibitor was used as starting material.

Spray dried protein is dissolved in 25 mM Tris buffer pH 8 providing a 10-5030 g/L protein solution, which is adjusted to a conductivity of 10 mS/cm with NaCl. The solution is stirred for 45 min and centrifuged at 4000 rpm 15 min.

Dissolved protein is loaded on an anion exchange column in an amount of 1-3 column volumes with a flow of 12 ml/min. The column is washed with 10% buffer elution buffer having a high salt content giving a conductivity of 10 mS/cm.

Elution is done with Tris pH 8 buffer with 1 M NaCl in one step going to 30% elution buffer having a high salt content.

NaCl is added to the initial protease inhibitor isolate (PI) and the initial patatin isolate to reach a conductivity of 80-120 mS/cm for the initial patatin isolate and 80-150 mS/cm for the initial protease inhibitor isolate.

Both fractions (the initial protease inhibitor isolate (PI) and the initial patatin isolate) are centrifuged at 4000 rpm 15 min and the isolates are loaded onto two different hydrophobic materials substituted with butyl groups. A content of 1-4 column volumes are loaded at a flow rate of 1.2 ml/min.
The bound proteins (the protease inhibitor isolates (PI) and the patatin isolates) are eluted stepwise from 100% high salt pH 8 buffer to pure water. 3 fractions comprising either protease inhibitor isolates and 2 fractions comprising patatin isolates are collected at medium (100-20 mS/cm) and low conductivity (< 20 mS/cm), respectively, from each of the different hydrophobic materials.

Coloured compounds will elute at high conductivity.

The resulting protease inhibitor isolates (PI) and patatin isolates are concentrated with 3-10 kD molecular weight cut off values using ultra-filtration filters and then subjected to diafiltration with water.

The resulting concentrated protease inhibitor isolates (PI) and concentrated patatin isolates dried, using spray drying.

References

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Claims

1. A method for obtaining at least two patatin isolates and at least two protease inhibitor isolates, said method comprises the steps of:

(v) providing fruit juice;

(vi) subjecting the fruit juice to an initial fractionation process obtaining an initial patatin isolate and an initial protease inhibitor isolate;

(vii) subjecting the initial patatin isolate to a chromatographic separation material providing a first patatin isolate and a second patatin isolate;

(viii) subjecting the initial protease inhibitor isolate to a chromatographic separation material providing a first protease inhibitor isolate and a second protease inhibitor isolate;

wherein the chromatographic separation material is selected from a hydrophobic material, for performing hydrophobic interaction chromatography (HIC), or a reversed phase, for performing reversed phase chromatography (RPC), preferably, the chromatographic separation material is a hydrophobic material.

2. The method according to claim 2, wherein the fruit juice is obtained from potato, preferably potato tubers (Solanum tuberosum).

3. The method according to anyone of claims 1-2, wherein the pH of the fruit juice is adjusted to a pH in the range of 7-10, such as in the range of 7.5-9.0, e.g. in the range of 7.7-8.5, such as about pH 8.0.

4. The method according to anyone of claims 1-3, wherein the fruit juice is adjusted to a conductivity of less than 50 mS/cm, such as less than 40 mS/cm, e.g. less than 30 mS/cm, such as less than 25 mS/cm, e.g. less than 20 mS/cm, such as less than 15 mS/cm.

5. The method according to anyone of claims 1-4, wherein the fruit juice is subjected to centrifugation or filtration before the initial fractionation of the fruit juice in step (ii).
6. The method according to anyone of the preceding claims, wherein the initial fractionation process is a chromatographic separation process, a filtration process, a centrifugation process, precipitation process or a combination hereof.

7. The method according to claim 6, wherein the chromatographic separation process is selected from the group consisting of an ion exchange chromatographic process, a mixed mode chromatographic process, an affinity chromatographic process or a combination hereof, preferably the chromatographic separation process is an ion exchange process.

8. The method according to claim 7, wherein the ion exchange chromatographic process is an anion exchange chromatographic process.

9. The method according to anyone of claims 7 or 8, wherein the chromatographic separation process involves a chromatographic separation material.

10. The method according to anyone of the preceding claims, wherein the initial patatin isolate is retained during the initial fractionation process and/or wherein the initial protease inhibitor isolate is found in the run through fraction.

11. The method according to claim 10, wherein the initial patatin isolates retained are eluted by addition of a first elution buffer.

12. The method according to claim 11, wherein the first elution buffer comprises 0.5-2M salt, such as 0.75-1.5M salt, e.g. about 1M salt and/or the elution buffer has a pH in the range of 7-10, such as in the range of 7.5-9.0, e.g. in the range of 7.7-8.5, such as about pH 8.0.

13. The method according to claim 12, wherein the salt is NaCl.

14. The method according to anyone of claims 11-13, wherein the elution is performed in a two step elution method.

15. The method according to anyone of the preceding claims, wherein the initial patatin isolate and/or the initial protease inhibitor isolate may be concentrated, preferably by ultra-filtration, nano-filtration and/or micro-filtration.

16. The method according to anyone of claims 1-15, wherein the conductivity of the initial patatin isolate is adjusted to a conductivity in the range of 60-170 mS/cm, such as 100-
130 mS/cm and the conductivity of the initial protease isolate may be adjusted to a conductivity in the range of 60-170 mS/cm, such as 110-140 mS/cm.

17. The method according to anyone of claims 1-16, wherein the initial patatin isolate and/or the initial protease isolate is subjected to a concentration treatment prior to the conductivity adjustment.

18. The method according to claim 17, wherein the concentration treatment comprises ultra-filtration, nano-filtration and/or micro-filtration.

19. The method according to claims 1-18, wherein the hydrophobic material comprises a ligand selected from the group consisting of alkyl-groups or aryl-groups.

20. The method according to anyone of claims 1-19, wherein the hydrophobic material comprises a ligand selected from the group consisting of butyl-groups; octyl-groups and phenyl-groups.

21. The method according to anyone of claims 1-20, wherein the at least two patatin isolates and/or the at least two protease inhibitor isolates are eluted from the chromatographic separation material by the addition of a second elution buffer.

22. The method according to anyone of claims 1-21, wherein the at least two patatin isolates and/or the at least two protease inhibitor isolates are eluted from the chromatographic separation material by using a stepwise elution wherein the salt content is changing from a high salt content to a low salt content during the elution.

23. The method according to claim 22, wherein the high salt content is a salt content in the range of 0.5-3M salt, such as in the range of 1-2M, e.g. in the range of 1.25-1.75M.

24. The method according to claim 22, wherein the low salt content is a salt content below 0.5M, such as below 0.3M, e.g. below 0.1M, such as below 0.05M, e.g. below 0.01M, such as below 0.005M.

25. The method according to anyone of claims 21-24, wherein the second elution buffer has a pH value in the range of 5.0-10, such as in the range of 5.5-9.5, e.g. in the range of 6.0-9.0, such as in the range of 7.0-8.5, e.g. about pH 8.0.
26. The method according to anyone of the preceding claims, wherein at least two patatin isolates and the at least two protease inhibitor isolates are concentrated, e.g. by using ultra-filtration, dia-filtration, nano-filtration, and/or micro-filtration.

27. The method according to anyone of the preceding claims, wherein the at least two patatin isolates and/or the at least two protease inhibitor isolates are dried by spray drying, freeze drying, or vacuum drying, preferably by spray drying.

28. A patatin isolate obtainable by the method according to anyone of claims 1-27.

29. The patatin isolate according to claim 28, wherein the proteins are substantially native proteins.

30. A protease inhibitor isolate obtainable by the method according to anyone of claims 1-27.

31. The protease inhibitor isolate according to claim 30, wherein the proteins are substantially native proteins.

32. A food product comprising the patatin isolate according to anyone of claims 28 or 29 and/or a protease inhibitor isolate according to anyone of claim 34 or 35.

33. Use of a patatin isolate according to anyone of claims 28 or 29, or a protease inhibitor isolate according to anyone of claims 34 or 35, in a food product or a feed product.

34. Use of a patatin isolate according to anyone of claims 28 or 29, or a protease inhibitor isolate according to anyone of claims 34 or 35, as a foaming agent, a gelling agent, or an emulsifying agent.
Abstract

The present invention relates to a method for obtaining at least two patatin isolates and at least two protease inhibitor isolates, said method comprises the steps of: (i) providing fruit juice; (ii) subjecting the fruit juice to an initial fractionation process obtaining an initial patatin isolate and an initial protease inhibitor isolate; (iii) subjecting the initial patatin isolate to a chromatographic separation material providing a first patatin isolate and a second patatin isolate; (iv) subjecting the initial protease inhibitor isolate to a chromatographic separation material providing a first protease inhibitor isolate and a second protease inhibitor isolate; wherein the chromatographic separation material is selected from a hydrophobic material, for performing hydrophobic interaction chromatography (HIC), or a reversed phase, for performing reversed phase chromatography (RPC), preferably, the chromatographic separation material is a hydrophobic material.