Dry fractionation for sustainable production of plant protein concentrates

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This research was conducted under the auspices of the Graduate School VLAG (Advanced studies in Food Technology, Agrobiotechnology, Nutrition and Health Sciences).

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Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Friday 6 March 2015
at 4 p.m. in the Aula.

Pascalle J.M. Pelgrom
Dry fractionation for sustainable production of plant protein concentrates
202 pages.

PhD thesis, Wageningen University, Wageningen, NL (2015)
With references, with summaries in Dutch and English

ISBN 978 94 6257 235 5

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1 General introduction

This chapter is part of a review publication and is submitted as: Schutyser, M.A.I., Pelgrom, P.J.M, van der Goot, A.J., Boom, R.M. (2015) Dry fractionation for sustainable production of functional legume protein concentrates.

1.1 Introduction

1.1.1 From protein isolates to dry-enriched protein fractions

The global demand for protein-rich foods is expected to double in the coming decades due to the increasing prosperity and world population. To keep up with the demand, the transition from an animal- to a plant-based protein supply is desirable from long-term economic and environmental points of view, as the production of animal protein imposes a severe burden on the available arable land, water and fossil fuels (Lqari, Vioque et al., 2002; Jayasena, Chih et al., 2010; Schutyser and Van der Goot, 2011). Besides, one kg of animal protein can only be obtained by feeding six kgs of plant protein (Pimentel and Pimentel, 2003). Furthermore, it has been estimated that, per MJ energy invested, 4 to 11 g animal protein are obtained, 8 to 57 g cereal protein and 41 to 77 g legume protein based on primary production and transportation (Pelgrom, Boom et al., 2015c). Therefore, legume proteins are an interesting alternative to animal proteins. However, current extraction processes to prepare plant protein isolates and subsequent plant-based food structuring (to produce e.g. meat replacers) involve copious amounts of energy and water, making these, at the moment, a less attractive alternative from the perspective of sustainability (Apaiah, Linnemann et al., 2006).

The conventional method to prepare plant protein ingredients is wet extraction (Figure 1). Legumes are a common source of protein as they have a high initial protein content (>20 g protein/100 g dry matter (Table 1)), dietary fibre content, contain a variety of micronutrients and phytochemicals, have a low level of fat (Messina, 1999), and are able to fixate nitrogen. Legumes can be divided into those that store energy as starch, and those that store energy as oil. Starch-rich legumes, such as peas and many beans, are dispersed in water to dissolve the protein and suspend the starch granules. Subsequently, the protein is recovered as a slurry by separating the starch granules in a hydrocyclone. Oil-rich legumes, such as soy and lupine, are subjected to a solvent extraction to remove the oil first. The defatted flour is then suspended in water and a suspension of protein and fibre is obtained. For both starch-rich and oil-rich legumes, solubilised proteins are separated from insoluble fibres at pH 9.

Proteins then get separated from soluble fibres by precipitating at their iso-electric point (pH 4.5 - 4.8). Subsequently, the pH is readjusted to 7 and a dry protein isolate is obtained after a final drying step (75-90 g protein/100 g dry matter) (Boye, Zare et al., 2010; Berghout, Pelgrom et al., 2015). It is clear that this wet process consumes large amounts water and chemicals (e.g. for acidification and neutralisation). Typically, for the production of lupine protein isolate from lupine seeds, more than 80 kg water/kg protein isolate is needed, 22.4 kg hexane/kg protein isolate, 0.04 kg NaOH/kg protein isolate and 0.04 kg HCl/kg protein isolate (Berghout, Pelgrom et al., 2015).

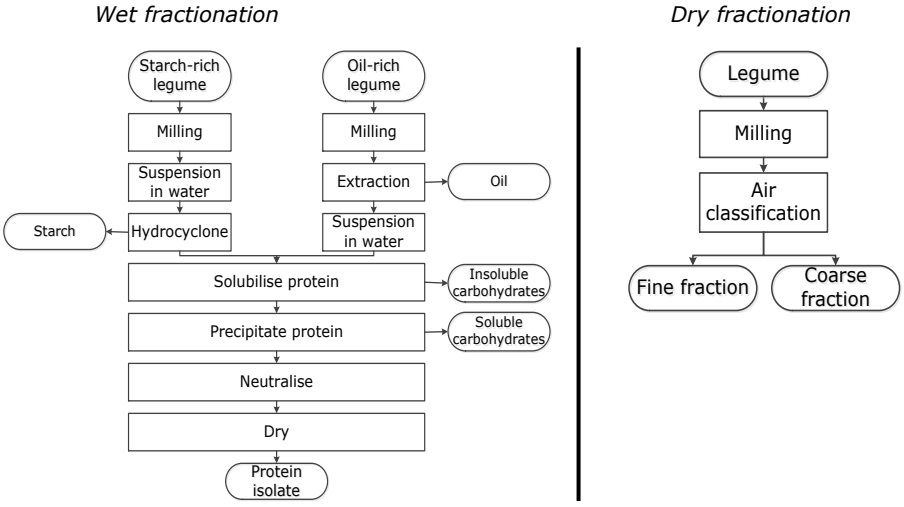


Figure 1 Schematic illustration of wet (left) and dry (right) fractionation process.

A more sustainable alternative to obtain protein-enriched fractions from legumes is dry fractionation (Figure 1), which uses milling and air classification. The addition of water is not necessary here and thus an energy-consuming drying step is not required. Another advantage is that the absence of a drying step together with the absence of chemicals retains the native functionality of components. Moreover, air classification is accredited for organic food production and the declaration of its products does not require E-numbers. The major

drawback of dry fractionation is the relatively modest enrichment in protein content that can be obtained compared to wet extraction.

Dry fractionation relies on the observation that milling can disentangle protein bodies and other cellular compounds into flour with particles of different composition. In starch-rich legumes, for example peas, cotyledon cells consist of starch granules (± 20 µm) embedded in a matrix of protein bodies (1-3 µm) surrounded by a fibre-rich cell wall (Tyler, 1982). Ideally, the starch granules are liberated during milling and the protein matrix is fragmented in particles smaller than 10 µm (Figure 2).

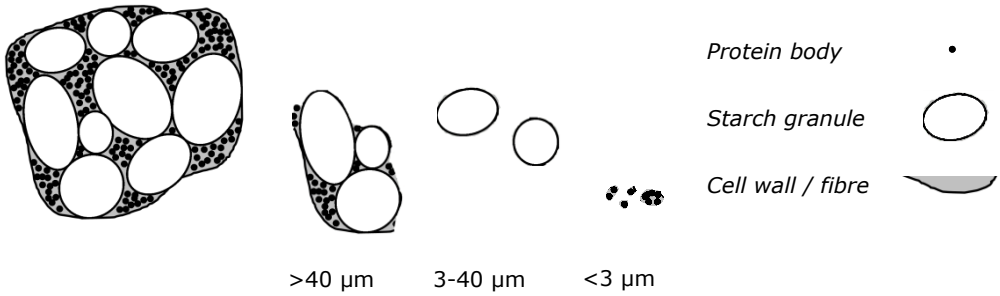


Figure 2 Schematic drawing of cells of pea and the fragments after milling.

The air classification after milling separates smaller protein-rich fragments from larger starch granules or fibre-rich particles. During this process, an air flow fluidizes the milled flour in a separation chamber. A classifier wheel submerged in the bed selects the small particles and allows these to form the fine fraction. Larger particles are rejected by the classifier wheel, leave the chamber at the bottom, and make up the coarse fraction.

Drag forces, created by the air flow, and centrifugal forces created by the classifier wheel, determine the size of the particles in the fine fraction. When these drag forces exceed the centrifugal forces, a particle can pass through the openings in the wheel and enter the fine fraction. By adjusting these parameters, protein concentrates have been produced from several legumes and

grains (Table 1). The protein content of the legume fine fraction varies between 49 and 70 g protein/100 g dry matter. The reason that a higher protein purity can be obtained with legumes than with grains, like wheat, is that the starch granules of legumes have a fairly uniform, large diameter (approx. 25-40 µm) compared to the mixed populations of small and medium-sized granules in most cereal grains (Vose, 1978). This difference in size (and weight) between starch granules and protein bodies is needed for air classification. For example, cow peas contain a high proportion of small starch granules, which results in a lower protein content of the fine fraction (Cloutt, Walker et al., 1987). Faba beans have large starch granules and can therefore be well separated (Tyler, 1984; Cloutt, Walker et al., 1987). Another factor that influences the separation efficiency of legumes is the amount of oil present. Chickpeas, lupine and soy have been reported to be unsuitable for dry fractionation (Sosulski and Youngs, 1979; Elkowicz and Sosulski, 1982).

Table 1 Protein enrichment by air classification of several legumes ± absolute deviation.

Legume / grain	Initial protein content (g/100 g dry matter)	Protein content fine fraction (g/100 g dry matter)
Wheat	12.3 ± 1.8	28.3 ± 4.0
Lima bean	23.7 ± 0.4	48.9 ± 0.8
Cowpea	27.2 ± 0.0	50.9 ± 0.2
Common bean	25.4 ± 1.1	55.2 ± 2.3
Navy bean	27.2 ± 1.6	56.7 ± 6.8
Lentil	23.2 ± 2.5	57.3 ± 5.3
Pea	23.8 ± 1.3	58.9 ± 3.0
Mung bean	27.2 ± 0.4	62.3 ± 1.2
Faba bean	31.0 ± 0.8	69.9 ± 5.2

(Jones and Halton, 1959; Kent, 1965; Stringfellow, J. et al., 1976; Vose, 1976; Sosulski and Youngs, 1979; Patel, Bedford et al., 1980; Tyler, Youngs et al., 1981; Aguilera, 1982; Elkowicz and Sosulski, 1982; Wright, Bumstead et al., 1984; Cloutt, Walker et al., 1987; Poel van der, Aarts et al., 1989; Wu, 1992; Bergthaller, Dijkink et al., 2001; Wu and Nichols, 2005)

1.1.2 Functional protein-enriched fractions to prepare attractive and healthy foods

Air classification of legumes has been investigated in the 1970s but since then received less attention. Major reasons for the renewed interest in dry fractionation are the wish for a more sustainable supply of protein-based foods and for processing routes that can deliver functional protein fractions to prepare attractive and healthy foods. Dry fractionation is a mild procedure providing the fully native functional protein. This is in contrast to wet extraction, during which proteins often denature because of pH shifts and excessive heat load during drying (Sosulski, 1987; Osen, Toelstede et al., 2014). The loss of solubility of the protein for example has a negative effect on foaming and emulsification properties of the ingredient (Wu and Inglett, 1974; Kinsella, 1976; Onimawo and Akpojovwo, 2006). Air-classified proteins retain their native functionality, but their properties may still be modified by mild heat treatment e.g. to improve water and fat binding capacity and gelation properties (Sosulski, 1987; Ma, 2011).

As discussed previously, air-classified enriched protein ingredients are less pure compared to wet extracted protein isolates and contain other major components such as starch and/or fibres. At first sight this could be regarded as a disadvantage. However, many traditional foods, e.g. bread, owe their attractive properties to the presence of, and interaction between, different constituents in the ingredients. Moreover, less refined ingredients are associated with health benefits compared to refined ingredients (Jacobs, Gross et al., 2009) and consumption of refined ingredients (protein, starch and lipids) incorporated into energy dense processed foods is strongly connected to the prevalence of obesity in the Western world (Rosenheck, 2008). Hitherto, air-classified pea protein has been successfully applied to replace egg protein in pasta, cakes and cookies (Nielsen, Sumner et al., 1980; Bahnassey and Khan, 1986). Other common applications of protein concentrates are meat and sausage substitutes and soups (Swanson, 1990; González–Pérez, 2009).

As indicated by Schutyser and Van der Goot (2011), to further increase the protein purity and use of dry separated fractions, more understanding of the

limiting factors in dry fractionation is needed. The milling behaviour that should lead to disentanglement between protein bodies and other cellular components should be improved by increasing our knowledge on the morphology of the seed and its breakage behaviour upon impact. Moreover, knowledge of material properties is the key to expand dry fractionation to oil seeds, like lupine, and to design pre-treatments or employ combination of multiple driving forces to enhance protein purity of the protein fraction. The challenge for the classification is that it does not separate all particles with different sizes; especially reducing interactions between smallest particles (Van der Waals interactions) and between particles that contain some fluid constituent e.g. oil, could provide options for further improvement. Finally, understanding of the functional behaviour of dry separated fractions is required for their application in food products. Technological characteristics of these complex mixtures of components should be valued rather than their protein purity.

1.2 Research objective and outline of the thesis

The objective of the work described in this thesis was to increase our understanding of both the material properties of the legume seeds, and of the process conditions relevant to the combined milling and air classification of legumes, and from this explore more sustainable routes for functional ingredient protein fractions. Thus, amongst others, the relation between seed properties and break behaviour was analysed, optimal milling and air classification conditions were evaluated and the relation between composition and functionality was analysed and compared to conventional protein isolates. Pea and lupine were selected as main model raw materials.

Chapter 2 presents a systematic study of milling and air classification of pea. The functional properties of air-classified fractions were evaluated based on their water holding capacity. The degree of denaturation and the composition were found to be important.

Chapter 3 describes detailed investigations of the breakage behaviour of pea and the disentanglement of its components as a function of the moisture content

and temperature. Glass transition curves were obtained and single pea fracture experiments were performed in the glassy and rubbery state.

Chapter 4 explores the functionality of air-classified pea fractions. Starch-rich fractions were gelatinized upon heating and protein-rich fractions were gelatinized by enzymatic crosslinking. Besides, natural occurring phase separation upon suspension led to the development of a new separation technique.

Chapter 5 analyses the air classification of lupine and functionality of its fractions. Lupine seeds have a different morphology and contain lipids, which ask for a different approach. The dispersibility of flours was evaluated and the functionality was assessed in terms of foam stability, viscosity and digestibility.

Chapter 6 aims at improving the protein content and yield of air-classified pea and lupine fractions by applying pre- and post-treatments. The effect of moisture content on the breakage behaviour of single peas found in chapter 3 was confirmed during pilot-scale milling experiments. Other pre-treatments that we analysed were defatting, freezing cycles and soaking. Electrostatic separation was investigated as post-treatment and enriched air-classified fractions further in protein content.

Chapter 7 presents factors that determine dry fractionation of starch-rich legumes. Optimal disentanglement was obtained for pea, bean, lentil and chickpea when the particle size distribution curve of flour and isolated starch granules maximally overlap. The protein content of the fine fraction was however legume-specific and could be explained by differences in particle density, seed hardness, starch granule size, fat content and flour dispersibility.

Chapter 8 provides a general discussion and gives an evaluation and an outlook on the future perspective of dry fractionation. The three themes of this thesis, i.e. increase knowledge on legume morphology, sustainability and functionality, were discussed.

2 Dry Fractionation for Production of Functional Pea Protein Concentrates

This chapter has been published as: Pelgrom, P.J.M., Vissers, A.M., Boom, R.M., Schutyser, M.A.I. (2013). Dry Fractionation for Production of Functional Pea Protein Concentrates. Food Research International, 53 (1), 232-239.

2.1 Abstract

Dry milling in combination with air classification was evaluated as an alternative to conventional wet extraction of protein from yellow field peas (*Pisum sativum*). Major advantages of dry fractionation are retention of native functionality of proteins and its lower energy and water use. Peas were ground by impact (ZPS50) and jet milling (AFG100) at various classifier wheel speeds to provide pea flours with different particle size distributions, protein contents and damaged starch levels. Peas were milled under various conditions to maximally disentangle starch granules from the surrounding protein bodies. The optimal milling conditions were confirmed by particle size analysis and Scanning Electron Microscope imaging. Too extensive milling, e.g. using ultrafine impact or jet milling, resulted in very fine flours (with $D_{0.5}$ < 10 µm) with poor flowability, whereas ultrafine jet milling led to an increased percentage of damaged starch. Subsequently, air classification was applied to separate small fragments (primarily protein bodies) from the coarse fraction (starch granules) to obtain enriched protein concentrates. Protein concentrates were obtained with protein contents between 51 and 55 g protein/100 g dry matter and a maximum protein recovery of 77%. Deviating cut-off size for air classification could be ascribed to build-up of material between the vanes of the classifier wheel. Finally, water holding capacity (WHC) tests were used to evaluate the functional properties of the pea protein concentrates. A liquid pea concentrate comprising 26 g protein /100 g sample of protein could be prepared from dry pea concentrates containing more than 30 g pea protein/100 g dry matter. This was explained by the high solubility of pea protein in its native state. After heat treatment of pea protein concentrates, a gel with a high WHC of 4.8 g water/g flour was obtained, which decreased with increasing protein content. Functional properties of the pea protein concentrates are interesting for preparation of high-protein foods or for replacement of egg protein functionality.

2.2 Introduction

Pea protein isolate is used to improve the texture and the nutritional quality of food products (Shand, Ya et al., 2007) and commonly produced by wet fractionation. Wet extraction starts with dispersion of pea flour in water after which the proteins dissolve and the starch granules are suspended. A hydrocyclone is used to separate the starch granules from the protein solution. The solubilised proteins are then precipitated at their iso-electric point (pH 4.8). Subsequently, the pH is readjusted to 7 and a dry protein isolate is obtained with a final drying step (75-90 g protein/100 g dry matter) (Boye, Zare et al., 2010). The major drawback of wet fractionation is partial loss of the native functionality of the proteins due to the pH shifts and drying. Moreover, this process uses many chemicals and a lot of energy and water (Schutyser and Van der Goot, 2011). It also excludes insoluble proteins from the isolate, which are generally highly aggregated proteins with specific functionality.

Dry fractionation by fine milling with subsequent air classification is a more sustainable alternative to wet fractionation for peas and several other legumes and grains (Bergthaller, Dijkink et al., 2001). After fine milling, the larger starch granules (20 µm) are physically detached from the smaller protein-rich particles (1-3 µm), which allows separation (Tyler, Youngs et al., 1981). Too coarse milling however leads to the presence of aggregates of protein bodies, starch granules and other cell components, which does not allow subsequent separation. Too fine milling leads to extensive starch damage and affects separation negatively, as the starch granule fragments and protein bodies have similar sizes. During air classification, the smaller protein-rich particles are separated from the larger starch granules based on size, shape and density (Boye, Zare et al., 2010). The protein content of the fine fraction depends on the initial protein content of the flour, the dispersibility of the flour and the cut point (Reichtert, 1982; Dijkink, 2007). The cut point is the size at which a particle has a 50% chance to move either to the fine fraction or to the coarse fraction. It can be adapted by selection of the appropriate air classification conditions, such as the classifier wheel speed and the air flow (Cloutt, 1986). Complete separation of protein from starch is hindered by protein that still

adheres to the starch granules after milling (Vose, 1978). A second milling step can be applied to increase the protein yield. However, a side effect is that more damaged starch will be present in the second protein fraction, decreasing purity (Tyler, Youngs et al., 1981).

The pea protein concentrates are used in food products, like meat and sausage substitutes and soups, for their solubility, water and fat binding capacity, gelation, foaming and emulsification capacity (Swanson, 1990; González–Pérez, 2009). After a heat treatment the water and fat binding capacity and the gelation properties are improved, while the solubility is decreased (Sosulski, 1987; Ma, 2011).

This paper presents a systematic study of milling and air classification for producing pea protein concentrates in combination with their functional analysis. Jet and impact milling were investigated to obtain pea flours with different properties (e.g. disentanglement behaviour, protein concentration) and particle size distributions. Subsequently, air jet sieving and especially air classification were used to prepare pea flour fractions enriched in protein. Air classification was carried out under different conditions to change the cut point and thus the protein shift. The air classification operation was verified with the model described by Bauder, 2004. Finally, the functionality of the protein concentrates was evaluated based on their water holding capacity, which is an important property of concentrates in many different food applications (Ma, 2011). In the functional analyses, the pea protein concentrates are compared to denatured pea protein concentrates.

It is hypothesized that both the degree of denaturation and the composition of pea concentrate mixtures affects their functionality (Schutyser and Van der Goot, 2011). Previous studies did not investigate the influence of the composition of an isolate on its functionality, but only focused on maximising protein content (Sosulski and Youngs, 1979; Wright, Bumstead et al., 1984). Retention of native functionality by prevention of denaturation is expected to increase the solubility of pea proteins (Alonso, 2000). The presence of residual

starch may have a positive effect on water holding capacity absorption (Sosulski and Youngs, 1979; Horvath, Ormai-Cserhalmi et al., 1989; Damodaran, 2008).

2.3 Materials and methods

2.3.1 Materials

Pre-dried yellow peas, *Pisum sativum*, were purchased from Alimex (The Netherlands). The producer's specifications of the yellow peas were: moisture 10-15 g/100 g pea, protein 23 g/100 g pea carbohydrate 62 g/100 g pea (starch 44 g/100 g pea), fat 2 g/100 g pea and ash 3 g/100 g pea. Pea protein isolate (NUTRALYS® F85G) and pea starch isolate (PEA STARCH N—735) were supplied by Roquette (France).

2.3.2 Milling and air classification

A ZPS50 impact mill or an AFG100 fluidized-bed jet mill (Hosokawa-Alpine, Augsburg, Germany) were used for the milling experiments. In impact milling, size reduction is achieved through collisions between powder particles and the wall of the mill, whereas in jet milling, inter-particle collisions are responsible for size reduction. The impact mill speed only influences the milling time and energy use and was fixed for practical reasons at 8000 rpm. Both mills were equipped with an internal classifier wheel that allows fine particles to leave the grinding chamber, while coarse particles are recirculated. The air flow and the classifier wheel speed are the most important parameters in determining the final particle size of the milled flour. The applied classifier wheel speeds were 2500, 4000 and 8000 rpm. The air flow was kept constant at 52 m^3/h and the screw feeder was set at 2 rpm (circa 0.75 kg/h). Each milling experiment was duplicated with 1 kg of yellow peas.

The milled peas were air classified in an ATP50 classifier (Hosokawa-Alpine, Augsburg, Germany). In an air classifier, flour is taken up in the classifier chamber by air flow. Small and light particles will be taken higher than heavy and large particles. At the top, a classifier wheel with slits rotates. Small particles go through the slits. Larger particles leave the classifier at the bottom (Figure 3). The size of the particles that can pass the slits decreases with the speed of the classifier. The applied classifier wheel speeds were 5000, 6000,

8000, 10000 and 12000 rpm. The air flow was again fixed at 52 m^3/h. The screw feeder rate was set at 20 rpm (circa 1 kg/h). This rate was not varied as it is generally accepted not to influence the air classification (Wright, Bumstead et al., 1984). The peas were not de-hulled: the hull fibres were collected predominantly in the coarse fraction (Vose, 1976). Each air classification experiment was duplicated with 500 g of flour.

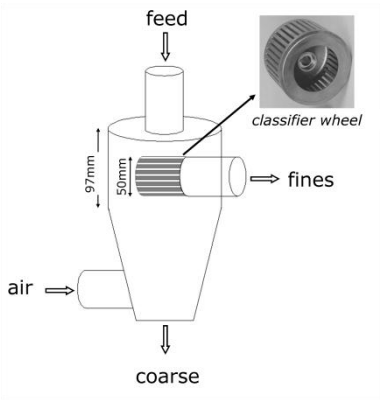


Figure 3 Schematic overview of the air classifier used in this study (ATP50).

The air classification process can be characterised by a cut point, which is the diameter of the particle that has equal chance to end up either in the fine or the coarse fraction. It can be varied by controlling the air flow and the classifier wheel speed. Cut points were determined experimentally on the basis of the particle size distributions and the yields of the fine, coarse and original flour fed to the air classifier. A Tromp curve was constructed to determine the cut point as a function of the classifier wheel speed (Leschonski, 1984):

$$T(x) = \frac{g * q_G(x)}{q_A(x)} \qquad \text{Eq. (1)}$$

in which x is the particle size, T(x) = 0.5 is the cut point, g is the weight of the coarse fraction divided by the sum of the weights of the coarse and the fine

fraction (-), $q_G(x)$ is the frequency distribution of the coarse fraction (-), $q_A(x)$ is the frequency distribution of the feed material (-).

Alternatively, the cut point can be approximated from the classifier configuration and the settings of the air classifier (Bauder, 2004). In the Hosokawa classifier, the separation is determined by the ratio between the centrifugal force created by the classifier wheel and the drag force of the sifter gas flowing through the wheel. For the fine particles, the drag force is dominant allowing them to pass the classifier wheel. For the coarse particles the centrifugal force is stronger than the drag force, which deflects them from the classifier wheel. The cut point is the particle size at which the drag force equals the centrifugal force (Bauder, 2004):

$$x = \frac{3}{4} c_w \frac{\rho_a}{(\rho_p - \rho_a)} \frac{v_r^2}{v_\varphi^2} r \qquad \text{Eq. (2)}$$

in which c_w is the drag coefficient (-), ρ_a is the air viscosity ($1.84*10^{-5}$ Pa s), ρ_p is the particle density (1400 kg/m^3), v_r is the radial velocity (m/s), v_φ is the tangential velocity (m/s) and r is the rotor diameter (50 mm). The radial velocity is determined by (Bauder, 2004):

$$v_r = \frac{Q_v}{2\pi\, r\, L} \qquad \text{Eq. (3)}$$

in which Q_v is the air flow (m^3/s) and L is the rotor length (26 mm). For cut points between 2 µm and 30 µm the following equation was used to estimate the drag coefficient (Bauder, 2004):

$$c_w = \frac{1}{3}\left(\sqrt{\frac{72}{Re}} + 1\right)^2 \qquad \text{Eq. (4)}$$

in which Re is the Reynolds number, which was determined for each combination of settings with iteration steps.

2.3.3 Air jet sieving

Various size fractions of the flours were prepared by air jet sieving (Alpine200 LS-N, Hosokawa-Alpine, Augsburg, Germany) with a 20 µm sieve at 4000 Pa for 2 min. Per analysis 9.8 g flour was sieved together with 0.2 g of aerosil®200 (Azelis Netherlands B.V., Oosterhout, The Netherlands) to improve the flowability. Aerosil was pre-sieved with a 20 µm sieve to enhance the anti-sticking effect.

2.3.4 Particle size distribution

The particle size distributions of all milled, sieved and air classified flours were determined in duplicate by laser diffraction using a Mastersizer 2000 equipped with the Scirocco 2000 dry dispersion unit (Malvern Instruments, Worcestershire, UK).

2.3.5 Scanning Electron Microscope

Scanning Electron Microscope (SEM) images of pea flour were obtained in a high-resolution field emission scanning electron microscope (Magellan 400 FEI, Eindhoven, The Netherlands). Conductive carbon cement (Plano GmbH, Wetzlar, Germany) was used to fix samples on SEM sample holders and to increase the electron conduction at the interface. The samples were dried and sputter coated with 15 nm iridium in SCD 500 (Leica, Vienna, Austria). The samples were analysed at 2 kV, at room temperature at a working distance of 4 mm. The contrast of the images was enhanced with Photoshop CS5 (Pelgrom, Schutyser et al., 2013).

2.3.6 Compositional and functional analyses

Dry matter content was determined by drying 2 gram of sample in an oven at 105ºC overnight.

The protein content was analysed using Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, Interscience). A conversion factor of N x

6.25 for pea protein was used. The measurements were done in duplicate. All protein contents reported are based on dry weight.

The starch damage was measured enzymatically using AACC method 76–31 (AACC, 2000) with a starch damage assay kit (Megazyme International Ireland, Ltd, Bray, Ireland). The analyses were performed on duplicate samples.

The WHC is a measure for the amount of water that a specific amount of flour can retain, and is mostly influenced by protein-water interactions, water-water interactions and physical capillary actions (Dahl, 1991). The water holding capacity (WHC) was determined as the amount of water that 5 g of material will retain after centrifugation at 2000 x g, using the drop-method (AACC, 1999). In this method just enough water is added to saturate the sample. Therefore, the measurement is not affected by the solubility of the sample. The proteins in pea flour were denatured by heating with a small amount of water for 1 h at 90ºC. All samples were measured in duplicate.

The sorption isotherms of pea protein isolate and pea starch isolate were determined with the Dynamic Vapour Sorption (DVS) Advantage apparatus (Surface Measurement Systems NA, Allentown, Pa., U.S.A.). Approximately 20 mg pea flour was weighed in a stainless steel mesh basket in the DVS equipment. The temperature was constant at 25ºC, and the starting relative humidity was set to 0% relative humidity (RH) for all experiments. When the mass of the flour changed at a pre-set rate of 0.0001%/min, the RH was programmed to change with 10% increments (5% for the last step) until the final RH of 95% was reached. The DVS Standard Analysis Suite v4.3 software for DVS Isotherm Analysis was used to calculate the sorption curve. The samples were measured in duplicate.

The relationship between the moisture content (M) on dry weight and the water activity (a_w) was described with the Guggenheim, Anderson and De Boer (GAB) equation (Anderson, 1946; De Boer, 1953; Guggenheim, 1966):

$$M=\frac{CkM_0a_w}{(1-ka_w)(1-ka_w+Cka_w)} \qquad \text{Eq. (5)}$$

in which M_0, k and C are the parameters. The parameters were obtained by least square minimisation of the difference between the measured moisture content and the predicted moisture content. The non-linear regression routine was implemented in Matlab® (The MathWorks, Inc., Natick, USA).

2.4 Results and discussion

2.4.1 Milling

Milling prior to dry fractionation should detach protein bodies from other cellular components like starch granules. Pea flours were prepared by impact and jet milling. Figure 4 shows the particle size distributions of the milled pea flours. Protein bodies are between 1-3 µm (Pernollet, 1978), starch granules are around 22 µm (Wright, Bumstead et al., 1984; Gujska, Reinhard et al., 1994; Al-Abbas, Bogracheva et al., 2006) and particles larger than 40 µm are whole cells or parts of cells (Vose, 1978). To facilitate protein enrichment, the peas should be ground to particles with a diameter of less than 40 µm to detach the protein from other cellular compounds. Less than 10% of the flour consisted of large particles (above 40 µm) at classifier speeds over 2500 rpm, therefore a single step milling procedure is sufficient (Vose, 1978; Wright, Bumstead et al., 1984). The Scanning Electron Microscope (SEM) images shown in Figure 5 visually confirm that the flour milled at 4000 rpm consisted of individual starch granules (S) and some cellular material (CM) with protein bodies. At a classifier wheel speed of 8000 rpm, the amount of particles in the size range between 17 µm and 26 µm decreased drastically: the starch granules were damaged generating fragments similar in size to those of the protein bodies.

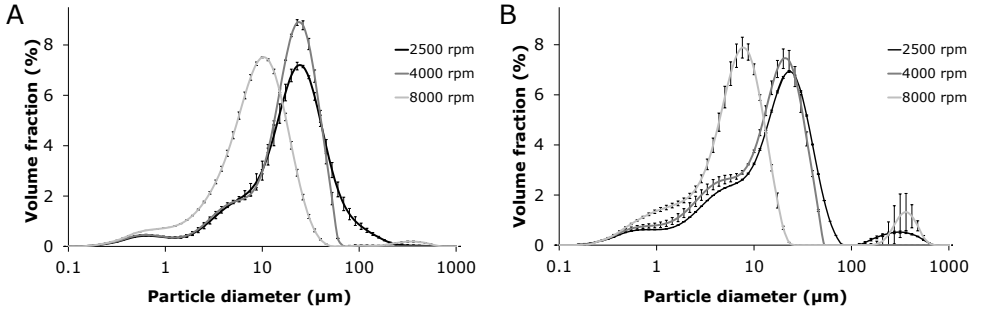


Figure 4 Particle size distribution of flour milled with the impact mill (A) and the jet mill (B) at various classifier wheel speeds. Error bars indicate absolute deviations.

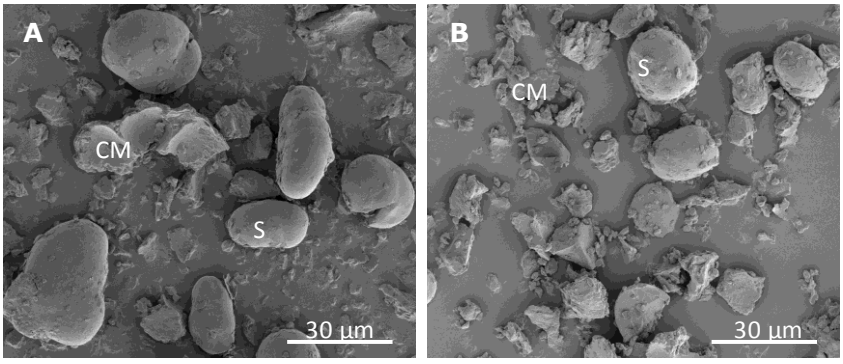


Figure 5 Scanning Electron Microscope (SEM) images of pea flour milled with the impact (4000 rpm) (left) or the jet (4000 rpm) (right) mill. Starch granules (S) and clusters of cellular material (CM) can be distinguished.

For both the jet and the impact mill the yield decreased with increasing classifier speed (Table 2). At the highest classifier speed, a very fine powder was obtained due to the increased residence time of the flour in the mill (Table 2). The increase in surface area and subsequent strong van der Waals force interactions caused poor cohesive flow behaviour of the powder and thus lower yields (Dijkink, 2007).

The protein content of the flours increased with increasing classifier wheel speed (Table 2). This indicated that pre-classification took place during milling. High classifier speeds allowed only particles in the size range of protein bodies to go

through the classifier wheel. The pre-classification was however not very efficient. The yield decreased drastically with increased protein content for both the impact and the jet mill (Figure 6). Yet, the impact mill covered a wider range of protein contents than the jet mill using the same classifier wheel and air flow settings.

The amount of damaged starch content was constant for the impact mill, but increased for the jet mill at high classifier wheel speeds (Table 2). The latter may be explained by the different milling mechanism leading to different fracture behaviour. Possibly, particle-particle collisions during jet milling are more powerful leading to fracture through individual starch granules, while during impact milling, fracture only occurs at weak lines in the cell tissue, e.g. at the interface of the protein and starch granule. The differences between the impact and the jet mill became more pronounced at higher classifier wheel speeds. In the jet mill, it was found that the formation of smaller particles was accompanied with more starch damage. This reduces not only the quality of the starch itself, but the starch fragments enter the fine fraction and thus reduce proportionally the protein content in the protein fraction compared to the impact mill at 8000 rpm (Table 2).

The moisture content of the flour decreased with increasing classifier wheel speed. The combination of the ambient air supply and the increasing residence time at higher classifier wheel speeds resulted in increased drying of the flour. It is reasonable to assume that the milling process was not affected by the change of moisture content, because the moisture contents and temperature of the flour kept the flour in the glassy state (Pelgrom, Schutyser et al., 2013).

The flours were treated with a 20 µm sieve to explore the separation potential of the protein from the other cellular components. Sieving provides only an indication because starch granules in peas can be smaller than 20 µm. Smaller sieves could not be used due to severe blockage. The difference in protein content between the two fractions obtained after sieving was largest for flours milled at 4000 rpm (Table 2). The separations achieved were similar for flours prepared with the impact and the jet mill. The flour milled at 2500 rpm had,

compared to flour milled at 4000 rpm, a higher protein content in the fraction >20 µm, which showed that the flour milled at 2500 rpm was not milled sufficiently to break the bonds between the protein bodies and the other cellular components. Little separation took place for the flours milled at 8000 rpm because the flour was over-milled.

For further classification experiments, milled pea flour was prepared using the impact mill at 4000 rpm. At this classifier wheel speed, small differences were found between flour milled with the impact and the jet mill. The impact mill was chosen for practical reasons.

Table 2 Milling results of yellow peas in an impact and a jet mill at different classifier wheel speeds ± absolute deviation.

Classifier speed (rpm)	Yield (%)	$D_{0.5}$ (µm)	Protein content (w/w%)	Damaged starch (% of starch)	Moisture content (%)	Protein content <20 µm (w/w%)	Protein content >20 µm (w/w%)
Jet mill							
2500	75.0 ± 1.0	15.8 ± 0.1	22.5 ± 0.3	2.3 ± 0.0	7.6 ± 0.1	26.2± 0.3	14.0± 0.6
4000	73.3 ± 0.2	12.9 ± 0.8	23.3 ± 1.9	3.4 ± 0.2	7.3 ± 0.3	27.0 ± 1.6	7.9 ± 0.1
8000	39.9 ± 2.1	6.1 ± 0.2	32.7 ± 2.0	20.7 ± 1.6	6.0 ± 0.1	30.8 ± 0.3	30.4 ± 0.3
Impact mill							
2500	86.5 ± 1.9	19.3 ± 0.8	22.4 ± 0.8	2.5 ± 0.2	10.1 ± 0.1	30.4 ± 0.8	15.3 ± 0.1
4000	72.8 ± 2.3	18.0 ± 0.5	24.3 ± 0.9	3.2 ± 0.5	8.2 ± 0.2	33.8 ± 0.9	12.7 ± 0.3
8000	15.5 ± 0.5	8.0 ± 0.1	55.4 ± 1.8	2.4 ± 0.0	7.9 ± 0.1	53.8 ± 0.3	50.0 ± 0.8

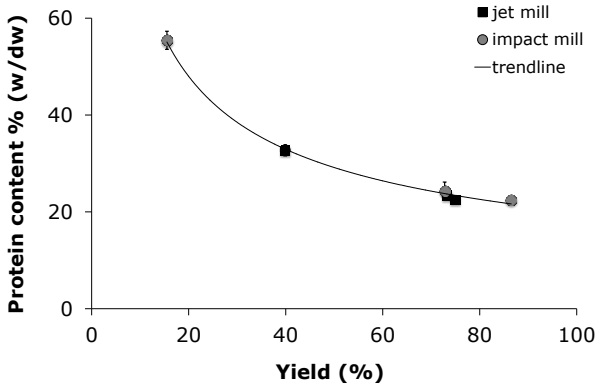


Figure 6 The protein content of the milled flours as function of yield for the impact and the jet mill. Error bars indicate absolute deviations.

2.4.2 Air classification

Size of the fractions

The pea flour was air classified at various classifier wheel speeds. As expected, the particle size of the fine fraction decreased with increasing classifier wheel speed (Table 3). Also the particle size of the coarse fraction slightly decreased with increasing classifier speed, which may be due to small particles adhering to larger ones and thus ending up in the coarse fraction (Dijkink, 2007).

The Tromp curve was used to calculate the cut point from the experimental data. The cut point decreased with increasing classifier wheel speed (Figure 7A). For all classifier speeds, the cut point was between the average size of the protein bodies (3 µm) and the average size of the starch granules (22 µm). This means that all used classifier speeds should achieve separation between protein bodies and starch granules.

The experimentally determined cut point was found to be higher than the cut point calculated theoretically with Equation 2 (Figure 7A). Visual inspection after classification showed that fouling occurred on the classifier wheel decreasing the surface between the vanes. Next to that, the model did not take into account the surface of the vanes in the classifier wheel. Therefore, the smaller surface of the classifier wheel was compensated for in the model by introducing an equipment

and sample specific fouling factor, α. In this factor α, the presence of the vanes was included next to the amount of fouling. The presence of the vanes was calculated to be approximately 25% of the total classifier wheel surface:

$$v_r = \frac{Q_v}{2\pi\, r\, L\, \alpha} \qquad \text{Eq. (6)}$$

Based on the difference between the theoretically calculated cut point and the experimentally determined one, the thickness of the fouling layer in between the vanes of the classifier wheel was estimated for each classifier speed (Figure 7B). The calculated fouling layer thickness values corresponded to visual observations that significant fouling occurred at increasing classifier wheel speeds, thereby increasing the cut point. For example, at a speed of 12000 rpm the surface between the vanes of the classifier wheel was reduced by one third due to the fouling layer.

Table 3 The influence of various classifier speeds on the average size of the fine and the coarse fraction, on the protein separation efficiency and on the protein shift ± absolute deviation.

Classifier speed (rpm)	D0.5 fine fraction (µm)	D0.5 coarse fraction (µm)	Protein separation efficiency (%)	Protein shift (%)
5000	8.8 ± 0.1	23.7 ± 0.3	76.8 ± 8.0	38.7 ± 5.1
6000	8.0 ± 0.3	23.0 ± 0.0	59.7 ± 3.7	32.3 ± 2.3
8000	6.2 ± 0.2	21.5 ± 0.1	43.7 ± 2.0	24.4 ± 0.9
10000	5.3 ± 0.1	20.3 ± 0.5	32.5 ± 2.0	18.2 ± 1.3
12000	4.8 ± 0.2	19.2 ± 1.4	29.2 ± 6.5	16.2 ± 3.5

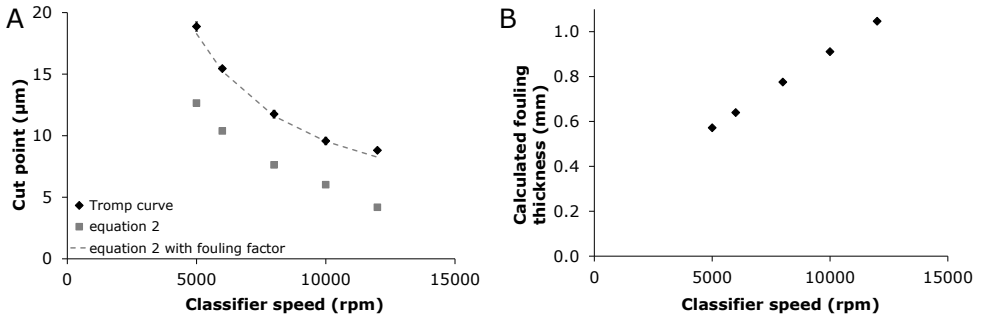


Figure 7 Cut point (µm) as a function of the classifier speed (rpm) calculated with the Tromp curve, Equation 2 and Equation 2 extended with the variable a. Figure 5B Calculated fouling layer thickness in the classifier wheel as a function of the classifier speed.

Composition of the fractions

Figure 8A shows the protein content of the fine, coarse and loss fraction. The loss fraction is the fraction that could not be simply removed from the system, i.e. fouling layer such as in the classifier wheel. The maximum protein content is in line with results from previous studies (Reichtert, 1978; Sosulski and Youngs, 1979; Tyler, Youngs et al., 1981; Wright, Bumstead et al., 1984; Bergthaller, Dijkink et al., 2001). The protein content in all produced fractions increased at higher classifier speeds because fewer particles could pass the classifier wheel. Therefore, more protein-rich particles ended up into the coarse and the loss fraction. The protein content of the coarse and the loss fraction was rather similar for each classifier speed. This indicated that the composition of a particle played a minor role in the chance that it would end up in the coarse or the loss fraction at a certain classifier wheel speed.

The protein yield was strongly dependent on the classifier wheel speed (Figure 8B). At higher classifier wheel speeds, the classifier wheel increasingly rejected smaller particles. Small particles had the tendency to stick to the wall, which led to formation of a fouling layer and thus large losses. Due to the formation of this fouling layer, the protein separation efficiency (PSE), which is the percentage of the total flour protein recovered in the protein fraction, and the protein shift, which is the percentage of protein that is shifted from the original flour into the protein fraction, decreased both (Table 3). For comparison, others report a

maximum protein separation efficiency of 67.6% (Tyler, Youngs et al., 1981) and a protein shift of 31.6% (Wu and Nichols, 2005) for peas.

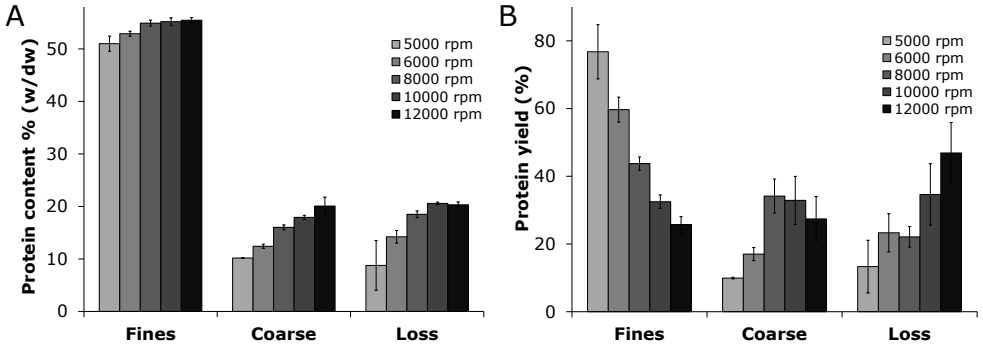


Figure 8 Protein content (A) and protein yield (B) of the fine, coarse and loss fraction of yellow pea flour classified at various classifier speeds. Error bars indicate absolute deviations.

Next to the protein content, the moisture content of the air classified samples was measured. The moisture content of the fine fractions was on average 7.3 ± 0.4 g moisture/100 g sample and the moisture content of the coarse fractions was on average 9.6 ± 0.7 g moisture/100 g sample, which corresponded to literature (Cloutt, 1986; van der Poel, 1989). An explanation for the lower moisture content of the fine fractions could be the smaller particle size of the fine fraction, which will lose moisture more quickly during the residence of the particles inside the system. Another factor that may play a role is the higher protein content in the fine fraction. Figure 9 shows that pea protein absorbs less moisture at the same RH compared to pea starch. For example, at a RH of 50% pea starch contains 10.4 ± 0.7 g water/100 g sample while pea protein contains 6.7 ± 0.4 g water/100 g sample. In Figure 9 the Guggenheim, Anderson and De Boer (GAB) equation was used to describe the moisture content (M) as a function of the water activity (a_w). For pea protein isolate the parameters at 25ºC were: M_0 4.68 ± 0.33, C: 8.72 ± 3.82, k: 0.90 ± 0.01. For pea starch isolate the parameters were: M_0: 7.69 ± 0.83, C: 12.48 ± 6.53, k: 0.78 ± 0.03. These values were in the same order of magnitude as found for pea seeds (Chen, 2003). The lower M_0 of pea protein compared to pea starch indicated that

pea protein contained less water in the monolayer, which is the water that is bound by hydrogen bridges at an a_w smaller than 0.3 (Fox, 1982).

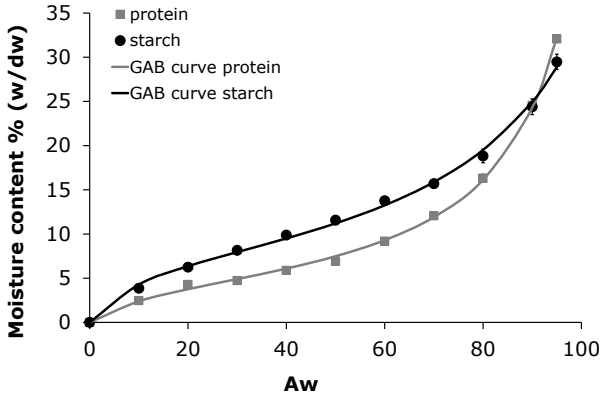


Figure 9 Experimentally determined sorption isotherms of pea protein isolate and pea starch isolate and their modelled GAB sorption isotherms. Error bars indicate absolute deviations.

2.4.3 Functionality

The functionality of the milled and air classified flours was evaluated based on their water holding capacity (WHC). The behaviour of air classified pea flour in water after centrifugation is dependent on the protein content (Figure 10A). At dry weight protein contents below 30 g protein/100 g dry matter, the WHC test yielded a supernatant and a pellet when sufficient water was added, which corresponds to the upper left region in Figure 10A. The WHC of the pellet increased with increasing protein content, analogous to a previous study (Sosulski, 1987). Samples with a higher protein content contained smaller particles as well as more small starch fragments, which both may contribute to a higher WHC. In literature WHC values of 0.72 g water/g flour for pea flour (15.2 g protein/100 g sample), 0.84 g water/g flour (52.4 g protein/100 g sample) (Sosulski and Youngs, 1979) and 1.03 g water/g flour (55.4 g protein/100 g sample) (Wang, 1999) are found. The variations in these results can be partially explained by differences in the methods to determine the WHC and natural variation between peas. In the samples that yielded a pellet and a supernatant

after centrifugation, a distinction was made between a starch layer, a protein layer and a water layer.

At flour protein contents above 30 g protein/100 g dry matter no supernatant was formed after centrifugation. Instead a homogeneous liquid concentrated sample was created, which can be related to the high solubility of native pea protein. The liquid concentrate comprised approximately of 25.7 ± 0.4 g protein/100 g solution, which is comparable to the solubility of non-denatured whey protein (Boye, 1995). This high solubility may potentially be explored for the preparation of liquid high-protein foods (Kinsella, 1976).

Pea flours with a different protein content were heated to evaluate the impact of denaturation on the WHC (Figure 10B). The concentrates having the lowest protein content had the highest WHC. Several authors (Sosulski and Youngs, 1979; Horvath, Ormai-Cserhalmi et al., 1989; Damodaran, 2008) explained this effect by the hydration capacity of pea starch, which is higher than pea protein. After denaturation by heating, the sample was either gelled or was separated into a supernatant and a pellet. The formation of a liquid concentrate was not observed anymore, which can be explained by the decrease in solubility due to denaturation of the proteins. The denaturation of the proteins and the gelatinisation of the starch increased the WHC significantly compared to that of the non-heated pea flour. Upon heating the subunits of the pea proteins are dissociated, which provides more water binding sites (Abbey and Ibeh, 1988; Owusu-Ansah and McCurdy, 1991). The WHC of denatured pea protein concentrate is very similar to that of denatured soy flour and soy protein concentrate (Wang, 1999) and to that of whey protein particles (Purwanti, 2012).

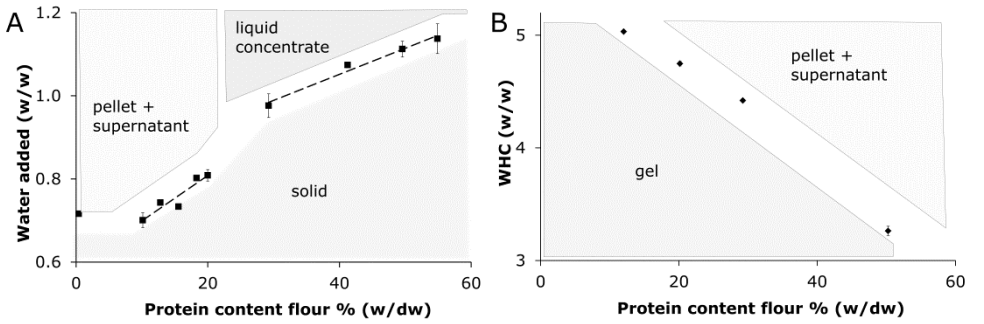


Figure 10 State diagram of native pea flour (A) with various protein percentages based on dry weight and WHC of denatured pea flour (B) with various protein percentages based on dry weight. Error bars indicate absolute deviations.

The results indicate that the pea protein concentrate prepared with dry fractionation has interesting functional properties. At protein contents above 30 g/100 g dry matter a concentrated, homogeneous liquid is formed. After heat treatment, a gel with a high WHC is obtained. Pea protein concentrates are thus well-suited for applications that require a significant WHC and for enhancing texture for example in baked goods and pastas (Sandberg, 2000). Moreover, mildly processed pea concentrates with high solubility may have potential for preparation of liquid high protein foods or replace egg protein functionality in specific applications.

2.5 Conclusions

Impact or jet milling at a classification speed of 4000 rpm led to optimum disentanglement of protein bodies and starch granules. Limited or no damaged starch was found using these milling settings. Milling at lower classification speeds led to incomplete disentanglement and too extensive milling, i.e. ultrafine jet milling (with D0.5 < 10 µm), resulted in increased starch damage and poor flowability of the flour. Air classification of pea flour milled at 4000 rpm yielded protein concentrates with a protein content of at least 51.0 ± 1.9 g protein/100 g dry matter with a protein recovery of at maximum 76.8 ± 9.4 %. Deviating cut-off size for air classification was related to build-up of material between the vanes of the classifier wheel. The thickness of this fouling layer

could be predicted from adapted equations describing cut-off size. Finally, functionality of the protein concentrates was evaluated on the basis of their water holding capacity (WHC). Pea protein concentrates made with dry fractionation could be used to prepare a liquid concentrate with 25.7 ± 0.4 g dissolved protein/100 g solution, on the condition that the protein concentration of the dry concentrate was more than 30 g protein/100 g dry matter. After heat treatment a gel with a high WHC could be obtained. For further research it would be interesting to look more deeply into the functionality of pea protein concentrates, for example to replace egg protein functionality in specific applications.

2.6 Acknowledgements

The authors wish to thank The PEAS Foundation and ISPT for financial support through the Intensified Protein Structuring project.

3 Thermomechanical morphology of peas and its relation to fracture behaviour

This chapter has been published as: Pelgrom, P.J.M., Schutyser, M.A.I., Boom, R.M. (2012). Thermomechanical morphology of peas and its relation to fracture behaviour. Food Bioprocess Technology, 6 (12), 3317-3325.

3.1 Abstract

Milling and subsequent air classification can be exploited for production of functional protein-enriched fractions from legumes and grains. Fracture behaviour is of large relevance to optimal disentanglement of protein and starch and is determined by the thermomechanical morphology of the seeds. Thermomechanical properties of peas were explored as a function of temperature and moisture content. Differential Scanning Calorimetry and Thermal Mechanical Compression Tests were carried out on pea protein and starch isolates yielding similar glass transition temperatures. Glass transition lines were successfully constructed using the Gordon-Taylor equation. Subsequently, three regions were identified in the state diagram; starch in the glassy and protein in the rubbery state, both components in the glassy state, and both components in the rubbery state. From single pea fracture experiments, it was found that the completely glassy peas fractured at a smaller critical compression distance compared to the peas in the other two regions. This can be explained by the elastic behaviour of the rubbery protein network, having a detrimental effect on the energy efficiency of milling processes. However, from Scanning Electron Microscopy it appeared that in rough fracture planes, visible when the protein was in the rubbery state, starch granules were present as more separate identities, suggesting increased disentanglement. Disentanglement of protein and starch by milling would then be optimal when protein is in the rubbery state. The latter can be achieved by milling at increased temperature and/or moisture content, which would be an attractive alternative.

3.2 Introduction

With the increasing world population and prosperity, the demand for protein-rich foods is expected to double in the coming decades. The transition from a livestock to a plant-based protein supply could lead to a more efficient food production (Aiking, 2011). Conventional production methods of plant-derived protein isolates usually involve wet fractionation, which involves copious amounts of water, uses significant amounts chemicals (e.g. for acidification and neutralization), and often results in large amounts of waste water. A less common method for production of protein concentrates, especially applicable to pulses and grains, involves dry milling and subsequent air classification. The major advantages of this method are higher energy efficiency and retention of native functionality of the proteins (Schutyser and Van der Goot, 2011). However, only limited protein concentration can be obtained with this method (e.g. 55% for field peas) because of the entanglement of the protein and starch. In field peas, the cotyledon, which is the largest part of the seed, consists of starch granules (53%) tightly embedded in a matrix with protein bodies (22%) (de Almeida Costa, 2006; Boye, Zare et al., 2010). Since milling enables protein bodies and starch granules to be disentangled, it is a critical step in the process of separation of these compounds. Essential to the success of dry fractionation is thus knowledge about the structural properties of the cotyledon and its corresponding milling behaviour (Tyler, Youngs et al., 1981; Tyler, 1984; Naguleswaran, 2010). The milling behaviour of legumes varies strongly with the mechanical properties of the seed and its constituents, since these determine the fracture behaviour. Structural tissue properties, such as cell morphology or presence of tissue layers, affect the fracture behaviour. This behaviour is also influenced by microstructural properties of the constituents, which are a function of the temperature and the moisture content. In dried pulses, starch granules and protein bodies are in the glassy state (Ballesteros, 2011), which is a non-equilibrium solid state (Abiad, 2009). Adding water or increasing temperature causes starch and proteins to change to the rubbery state. However, as proteins and starch exhibit different glass transitions, a region exists where proteins are in the rubbery state, while the starch granules are still glassy.

Numerous analysis methods can be used to measure glass transition in food ingredients. These methods can be classified into thermal (Differential Scanning Calorimetry), mechanical (Thermo Mechanical Analysis, Dynamic Mechanical Analysis), spectroscopic (Electron Spin Resonance, Nuclear Magnetic Resonance) and electrical (dielectric measurements) methods (Seyler, 1994). With DSC, the glass transition temperature is derived from the change in heat capacity between the glassy and the rubbery states. The mechanical and dielectric techniques measure a change in mobility of molecules around the glass transition. The spectroscopic techniques determine the glass transition based on information on chemical bonding and molecular mobility (Roos, 2010). The most common method is Differential Scanning Calorimetry (DSC) because it is an easy, rapid and reliable method. Several authors reported that DSC may not accurately measure the Tg in complex food systems (Boonyai, 2007; Shrestha, 2007; Sablani, 2010). For high molecular weight protein and carbohydrates, the change in specific heat capacity is small during glass transition. This small change causes broad and indistinct transitions. For these materials, detecting changes in the mechanical properties has proven to be more sensitive. A relatively new and promising test to measure this is the Thermal Mechanical Compression Test (TMCT), which is described by Boonyai (2007). The test is relatively easy and was shown to give accurate values for the glass transition in various dry food systems. It uses a probe, which applies a constant force on the sample under continuous heating. The glass transition temperature (T_g) is then characterised by displacement of the probe, resulting from increased mobility of materials in the rubbery state. The TMCT method has been compared with DSC and Thermal Mechanical Analysis and successfully applied to various dry food systems, such as skimmed milk powder (Boonyai, 2007), pasta (Rahman, 2011) and rice (Thuc, 2010).

In this study it is investigated whether specific combinations of moisture contents and temperature could provide different mechanical properties to starch and protein (e.g. due to glass transition) and thus lead to different fractures through cotyledon cells. This fracture behaviour is relevant for dry fractionation purposes, which requires complete disentanglement of protein bodies and starch granules. It is anticipated that the results support the design

of optimal pre-treatment and milling procedures. We report on freeze and glass transition lines of the cotyledon tissue constituents of field peas based on DSC and TMCT analyses of pea starch and protein isolates. Moreover, the measured glass transition temperatures are compared to the calculations following the Gordon-Taylor equation (Gordon, 1952; Sun, 1997). The data on glass transition are combined with experimental data on single pea fracture behaviour as characterised by stress-strain curves and Scanning Electron Microscopic (SEM) images. It should be emphasized that these single pea fracture experiments are only indicative for fracture during actual milling processes, as the forces and deformation rates applied during milling are of a different order of magnitude. Finally, it is discussed how single pea fracture behaviour is influenced by the microstructural properties at different temperatures and moisture contents. This discussion provides a basis for the further systematic exploration of milling and the dry fractionation process.

3.3 Materials and methods

3.3.1 Materials

Pre dried dehulled yellow peas, *Pisum sativum*, were purchased from Alimex (The Netherlands). Pea protein isolate (NUTRALYS® F85G) and pea starch isolate (PEA STARCH N—735) were obtained from Roquette (France). These isolates have been produced from pea flour via a wet fractionation process, in which a slurry is prepared from pea flour. The starch is then recovered by cyclone separation and subsequently dried. The pea proteins are coagulated from the slurry and spray dried.

3.3.2 Sample preparation

The moisture content of pea protein and starch isolates were varied to obtain glass transition data (5-20 g water/100 g sample) and to obtain freeze transition data (20-80 g water/100 g sample). Based on the DSC results, moisture contents were selected in combination with a specific temperature to obtain peas with protein and starch in rubbery and/or glassy states. Following, fracture experiments of these peas were carried out with a Texture Analyser. Peas and isolates were brought to the target moisture content (10% - 80%) by addition of

water followed by equilibration overnight. Peas and isolates with a target moisture content below 10% were placed in a climate chamber at 20ºC with a relative humidity of 5%. The relative humidity in the climate chamber was set according to the isotherm of the isolates. The isotherm was determined with the Dynamic Vapour Sorption (DVS) Advantage apparatus (Surface Measurement Systems NA, Allentown, Pa., U.S.A.). The final moisture content of all samples was determined in duplicate by measuring the weight loss after overnight drying at 105ºC.

3.3.3 Thermal transition by DSC

Differential Scanning Calorimetry (DSC) measurements were performed using a Diamond DSC (PerkinElmer). The glass transition temperature was measured for pea protein isolates and pea starch isolates at various moisture contents. About 20–25 mg of sample was weighed in a DSC stainless steel pan. The DSC analyser was calibrated using indium, and an empty stainless steel pan was used as a reference. During the measurement, nitrogen was used as carrier gas. Samples were cooled to -60ºC at a rate of 100ºC/min followed by a heating ramp of -60ºC to 160ºC at a rate of 10ºC/min. The heating rate was chosen based on literature (Casey, 1982; Bora, 1994; Ratnayake, Hoover et al., 2001; Simsek, Tulbek et al., 2009). A second cooling step at a rate of 100ºC/min to -60ºC and a second heating step from -60ºC to 160ºC were applied to obtain a clearer glass transition. Each sample was prepared in duplicate and each of these duplicates was measured twice. Measurements were analysed for the glass transition midpoint using Start Pyris Software.

3.3.4 TMCT method

The Thermal Mechanical Compression Test (TMCT) device is composed of a concentric cylinder (20mm diameter, 40mm height) with a water chamber in the sidewalls and a solid bottom (Figure 11) for temperature control. A ramp of 5ºC to 80ºC was created by connecting the water chamber to a water bath (Julabo FP50-HE, Julabo, Seelbach, Germany). A heating rate of 3ºC/min was used. The temperature in the bottom of the cylinder was continuously recorded (Testo 175T3, Testo GmbH & Co., Lenzkirch, Germany). A 15mm cylindrical probe attached to a Texture Analyser (Instron-5564Series-Table-Model-Systems-Twin-

column-design, Canton USA) equipped with a 2000N load cell, exerted a constant force of 30N on 2g of sample. The force–displacement curve was measured with the Bluehill 2 Texture Profile Analysis software. For each sample, the force-displacement curve was corrected for thermal expansion of the equipment by subtracting the force-displacement curve of maltodextrin (Aldrich Chemical Co.; dextrose equivalent 13–17), which was chosen as reference because it has a high T_g value (T_g > 180°C), and it is physically and chemically stable. Skim milk powder (Sigma-Aldrich) was used to compare the set-up with the Thermal Mechanical Compression Test designed by Boonyai (2007). Each sample was measured at least in duplicate.

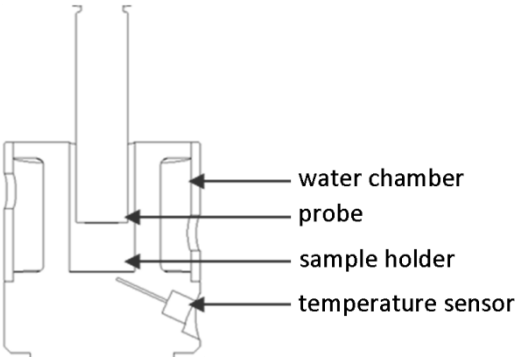


Figure 11 Schematic drawing of the TMCT sample cell.

3.3.5 Fracture behaviour analysis

The mechanical properties of pea cotyledons were determined with the same Texture Analyser and sample holder as used for the TMCT measurements Figure 11). The temperature was controlled by connecting the water chamber to a water bath (Julabo FP50-HE, Julabo, Seelbach, Germany). A dehulled and split cotyledon, flat-side down, was compressed between the cylindrical probe and the bottom-plate at a crosshead speed of 20mm/min till breakage occurred. The force-deformation curve was recorded with the Bluehill 2 Texture Profile Analysis software. For each treatment ten cotyledons were randomly selected and the average critical compression distances leading to fracture were reported.

3.3.6 Microscopic analysis with SEM

The Scanning Electron Microscope (SEM) images of yellow peas broken by Texture Analyser (TA) at various moisture contents and temperatures were obtained in a high-resolution field emission scanning electron microscope (Magellan 400 FEI, Eindhoven, The Netherlands). Conductive carbon cement (Plano GmbH, Wetzlar, Germany) was used to fix the samples on SEM sample holders and to increase the electron conducting at the interface. After the solvent evaporated, the dry samples were sputter coated with platinum (Jeol JFC 1200 fine coater Tokyo, Japan). The samples were analysed at 2kV room temperature and the images were contrast enhanced with Photoshop CS5.

3.4 Results and discussion

3.4.1 Thermomechanical characterization of pea protein and starch

Differential Scanning Calorimetry (DSC) thermal curves for pea protein and starch isolates were measured at a dry matter content of 20 g flour/100 g sample up to 95 g flour/100 g sample. The curves of both isolates showed a glass transition region instead of a sharp glass transition point. An explanation is that both protein and starch are not pure components, but a mixture of components, which exhibit a distribution of glass transition temperatures (Cuq, 2003). The glass transition temperature midpoint was determined and plotted in the state diagram given in Figure 12. The glass temperature decreases as the moisture content increases due to the plasticizing effect of water. Water works as a plasticizer because it increases the mobility of polymer chains (Levine, 1986). Peas that have a moisture content and temperature corresponding to the region below the glass transition points are in the glassy state. This means that dried peas at room temperature are glassy, as their dry matter content is typically around 90%. Peas are biologically in this quiescent state to survive dormancy (Ballesteros, 2011). At higher moisture contents peas are in the rubbery state at room temperature. Increasing the moisture content or the temperature induces a glass transition for both protein and for starch, but faster for the protein. This difference has been recorded for several sources of protein and starch in the literature (Cuq, 2003). The glass transition temperature for pea starch at similar moisture content is higher compared to pea protein

because the molecular weight of starch is larger than that of protein, which is in line with the data from Figure 12) (Colonna, 1984; Owusu-Ansah and McCurdy, 1991). A difference in glass transition temperature (T_g) for pea protein and pea starch is interesting as it means that the pea cotyledon has a state in which protein is already in the rubbery state while starch is still in the glassy state. At a dry matter content below 0.8 (kg dry matter/kg total) the phase transition (freezing) of the water present in the samples gives more pronounced peaks than the glass transition peaks and thus no data could be obtained on glass transition (Sman, 2007; Ballesteros, 2011).

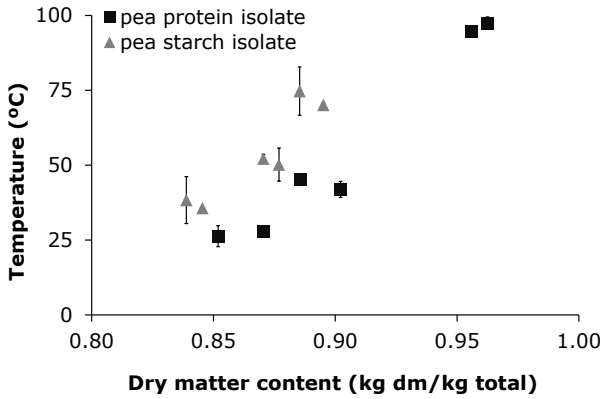


Figure 12 The glass transition temperatures of pea protein and starch isolates as a function of dry matter content (error bars indicate standard deviation).

The Thermal Mechanical Compression Test (TMCT) was used to confirm the T_g values measured with the DSC. The TMCT curves of pea protein (moisture: 13.9%) and starch (moisture 13.5%) isolates are shown in Figure 13. These curves were obtained by subtracting the compression of the reference from the compression of each sample, the reference being a material that remains in the glassy state. This reference will also undergo compression due to normal relaxation in the powder bed due to rearrangement of the powder particle. By subtracting this from the compression behaviour of the actual sample, the compression due to changes in its state is obtained. When the reference is compressed less during heating than a sample, the TMCT curve shows an increase. The reference can be compressed less compared to a sample because

the reference remains in the glassy state during the measurement while the sample undergoes a state transition. More compression is expected of a rubbery sample since molecules in the rubbery state have a higher mobility than molecules in the glassy state (Slade, 1991). In Figure 13, pea starch showed more compression than the reference: the curve increases with increasing temperature. This is the behaviour that is usually found with relatively simple materials (Boonyai, 2007; Rahman, 2011). The curve of pea protein isolate, however, shows first a compression, followed by an extension. The glass transition temperature of pea protein isolate coincides with the bending point of compression. We speculate that this unexpected behaviour is because of relaxation of the powder particles above the glass transition. The protein isolate particles are spray dried, and hence are quite porous. As soon as the particles become rubbery, the pores will collapse under the compression, and the sample will compress considerably. Thus it is reasonable to assume that the region where the sample curves start to deviate from zero is the glass transition region.

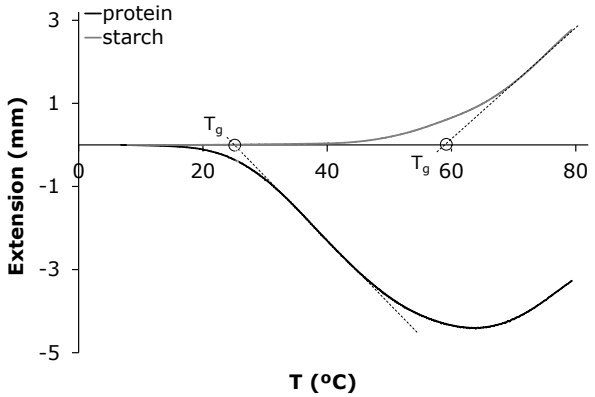


Figure 13 TMCT curves of pea protein and starch isolates. The extension of the probe, which applies a force of 30N on a sample, is plotted as a function of the increasing temperature of pea protein isolate (moisture:13.9%, T_g:25ºC) and pea starch isolate (moisture:13.5%, T_g:59ºC).

Figure 14 gives a comparison between the T_g values measured with DSC and TMCT. The isolates that were expected to have a T_g between 20ºC and 60ºC were measured. In this range the TMCT set-up was able to detect the T_g. For each measurement a new sample was made, therefore samples tested by DSC

and TMCT did not have exactly the same moisture content. The measurements of the TMCT strengthen the results obtained with the DSC. TMCT results can however differ from DSC results, because DSC monitors the sudden enthalpic and heat capacity change during glass-rubber transition whereas TMCT determines the exact point of mechanical deformation. Therefore, the T_g from TMCT can be expected closer to the stickiness point, which is usually several degrees higher than the T_g from DSC (Shrestha, 2007). As the TMCT measures changes at the particle surface the test can give lower Tg values than DSC (Boonyai, 2007). No differences were however found for the pea protein and starch isolates.

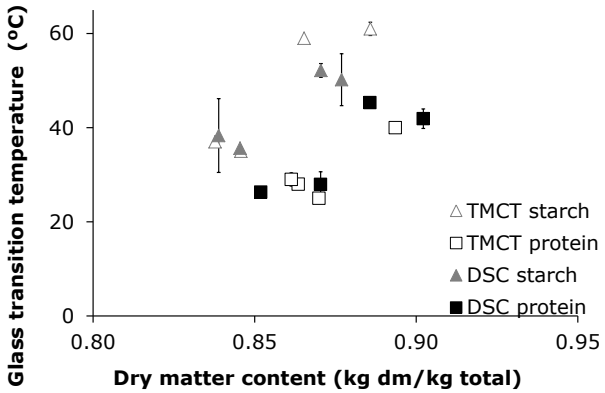


Figure 14 Comparison of the T_g for pea protein and starch isolates measured with DSC and TMCT (error bars indicate standard deviation).

The Gordon-Taylor equation is fitted to the measured glass transition temperatures of pea protein and starch isolates. This equation is commonly used to describe the effect of the water content on the glass transition temperature, T_g, of a specific component. Several other equations exist that can be used like, the Couchman-Karasz equation or the Kwei equation. The Gordon-Taylor equation is chosen because it has least parameters and has been successfully applied to seeds (Sun, 1997).DSC results are used for this purpose because the measured data range was larger than for TMCT. The Gordon-Taylor equation can be written as:

$$T_g = \frac{w_1 T_{g1} + k w_2 T_{g2}}{w_1 + k w_2}$$

where T_{g1} and T_{g2} are the glass transition temperatures of the water and pea isolates, respectively. T_{g1} is the glass transition of pure water and is taken to be 136K. This value is, however, subject of discussion (Velikov, 2001; Yue, 2004). Further, w_1 and w_2 are the weight fractions of water and pea isolates in the blend; k is a fitting parameter. Parameter k can be related to the strength of interactions of the sample components (Gordon, 1952).

The values of the parameters k and T_{g2} for the Gordon-Taylor equation were obtained by minimising the sum of squares of the differences between the measured T_g and the predicted T_g. The non-linear regression routine was implemented in Matlab®. It is noted that only dry matter contents above 0.8 (kg dry matter/kg total) were included for this because at lower dry matter contents, the freezing line of water interferes with the results. The freezing line of water is obtained by plotting a line through the freezing points of the water in the samples. The parameters and the state diagram are given in Table 4 and Figure 15, respectively. The values of the parameters of the pea protein and starch isolates found are in the same order of magnitude as those found for other plant-derived components Table 4).

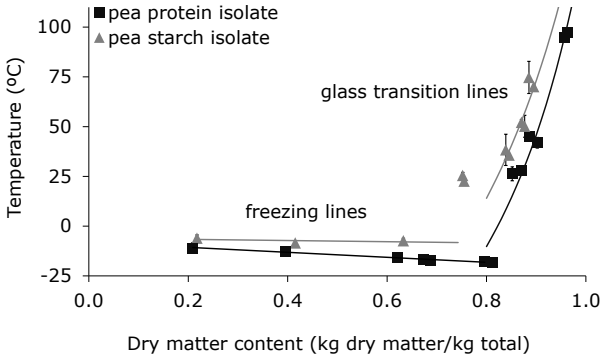


Figure 15 State diagram of peas based on DSC results of pea protein isolate and pea starch isolates (error bars indicate standard deviation).

Table 4 Tg and k values of several plant materials calculated with the Gordon-Taylor equation.

	k	T_{g2} (ºC)
Pea protein (this work)	5.06 ± 0.60	146.91 ± 11.55
Pea starch (this work)	4.18 ± 1.46	169.45 ± 40.09
Gluten (Micard, 2000)	5.10	173 ± 1.3
Gluten (Toufeili, 2002)	3.9 ± 0.24	127.6 ± 4.1
Corn-lentil mixtures (Lazou, 2011)	4.07 ± 0.19	169.04 ± 3.73
Bean and pea axes (Sun, 1997)	7.7	125
Tomato pulp (Goula, 2008)	6.69	139.65

3.4.2 Fracture behaviour of single peas in relation to protein-starch disentanglement

The constructed state diagram of pea protein and pea starch was used to analyse fracture behaviour of peas in different states. Based on the state diagram, the state of starch and protein in peas was changed by increasing the moisture content and / or the temperature. Figure 16 shows the effect of temperature (lower right graph) and dry matter content (upper right graph) on the critical compression distance leading to fracture. An increase in temperature or an increase in moisture content led to an increase in compression distance, which is in agreement with the literature and is explained by the elastic properties of the material (Dijkink, 2002; Altuntas, 2007; Łysiak, 2007). Peas with a low moisture content and /or low temperature showed one clear fracture line in the stress – strain curves obtained with the Texture Analyser (results not shown). Peas with a high moisture content and / or high temperature showed several small fracture lines in these curves as compression advanced. The critical compression distance was found to change between a dry matter content of 0.85 and 0.90 (kg dry matter / kg total). This was also reported by Dijkink (2002) and Łysiak (2007). Around a dry matter content of 0.87 (kg dry matter/ kg total) the glass transition of pea protein takes place. The compression of peas containing rubbery protein and glassy starch proceeds differently from that of

peas having glassy protein and glassy starch, which is explained by the appearance of the protein as a network around the starch granules. During the transition between the glassy and rubbery state for the starch no influence was observed on the critical compression distance. Results of experiments with peas with a dry matter content lower than 0.83 (kg dry matter/ kg total) are not shown because no fracture occurred, but only plastic deformation.

As could be expected it was observed that completely glassy peas showed clearly brittle fracture behaviour; with one impact the cotyledon was broken along a flat fracture surface. Rubbery peas showed more elastic fracture behaviour, indicating that during compression the cells were gradually torn apart. These mechanical properties are very relevant for the efficiency of a milling process. The energy input for size reduction of elastic materials is much higher than for brittle materials. In addition, creating smaller and smaller particles will become progressively more difficult because the weakest flaws will have been eliminated as the size is reduced, and the many microscopic fractures act as an energy sink during deformation (Coulson, 2002). Therefore, it is expected that ultrafine grinding of rubbery peas will be difficult or impossible during impact or jet milling. Instead, cryogenic milling is referred to as a method to increase brittleness of particles and thus grind materials more easily into an ultrafine powder (Hemery, 2011). However, if the fracture behaviour of the rubbery peas is such that the protein and starch are more disentangled, it could be acceptable that more energy is consumed for milling. This may for example coincide with the ambient milling of wheat bran, where ambient milling has been found to provide more tissue disentanglement of constituent layers with corresponding mechanical properties compared to cryogenic milling (Hemery, 2011). Still, it should be investigated whether the size reduction that can be achieved is also sufficient.

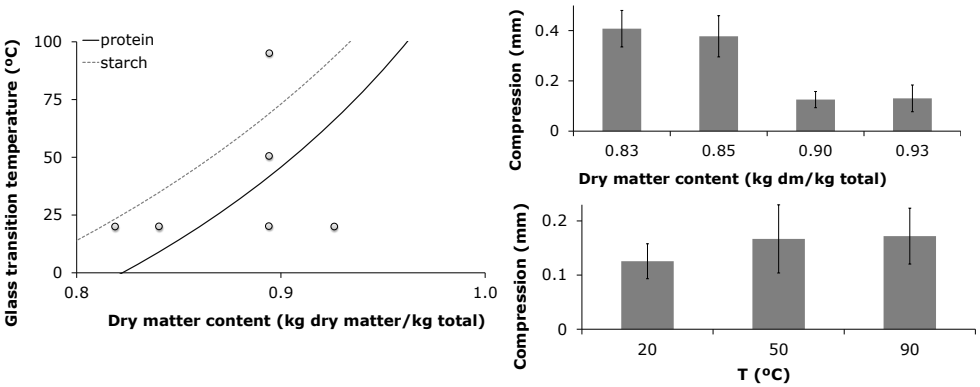


Figure 16 State diagram of peas. The circles in the state diagram represent the points in which samples are taken. On the right side the Texture Analyser results are plotted in which the critical compression distance is a function of temperature or moisture content (error bars indicate standard deviation).

Subsequently, we investigated the fracture surface of the peas broken with the Texture Analyser using Scanning Electron Microscopy (SEM). Images were taken of the cells of the inner pea cotyledon. These cells were selected to obtain uniformity and a representative view because the chemical composition and the microstructure of the inner and outer layers differs (Kosson, 1994; Otto, 1997).

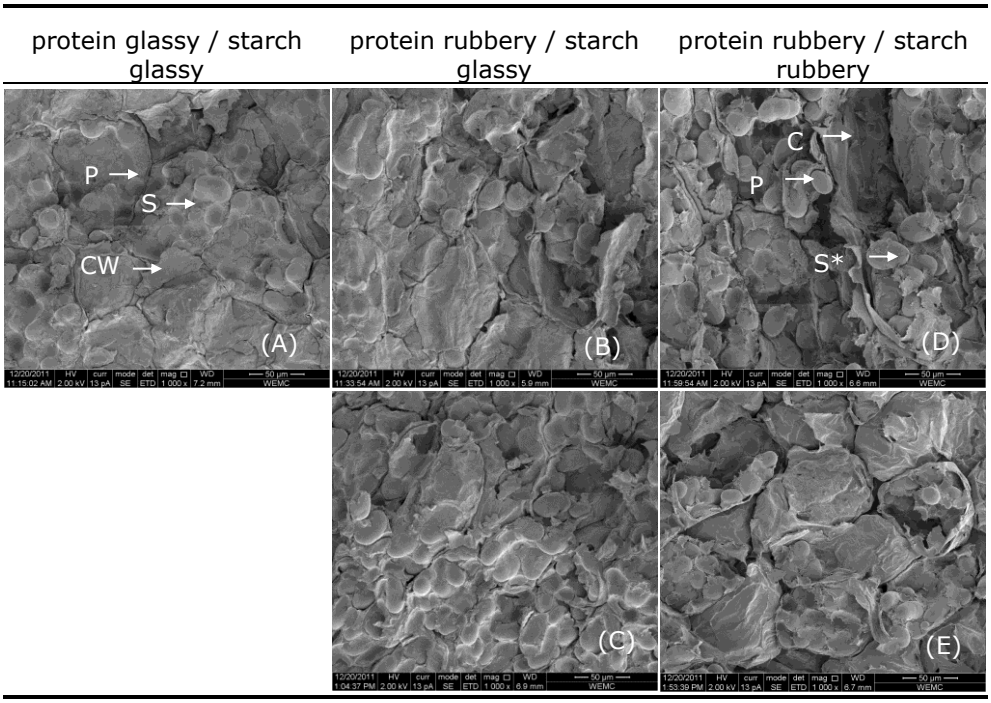
The microstructure of the fracture surface of dried glassy peas is shown in Figure 17A. Irregularly shaped cells form a compact matrix. Inside the cells, starch granules (53%) are tightly embedded in a matrix with protein bodies (22%). The typical size of a pea starch granule is 15 µm and the typical size of a pea protein body is 2 µm (Whitaker, 1977). In these cells both protein and starch were in the glassy state. The chemical bonding between starch granules and the protein matrix has been reported to be relatively weak in dry pea cotyledon cells (Parker, 2008). Fracture was therefore assumed to follow these contact lines, which was confirmed by parts of the granules emerging from of or by empty holes remaining in the fractured surface (Figure 17A). Fracture, however, also occurred in between cells.

Figure 17B and C show the fracture surface of peas with protein in the rubbery state. The fracture plane was rougher and irregular compared to that of peas

that were completely in the glassy state. Moreover, the starch granules seemed to be more disentangled from the protein matrix. Figure 17D and E show the break surface of pea cells in the rubbery state. Also in these images, the starch granules are more separate from the protein matrix compared to completely glassy cells. The fracture lines in these cells did not coincide with the contact lines between starch granules and protein matrix, but were throughout the whole cell. Similar fracture behaviour was observed for cowpeas after a heat treatment of 50-90ºC (Hung, 1990). Another observation from the SEM images was that cells in the rubbery state were less tightly ordered than cells in the glassy state. The cell wall of these rubbery cells had loosened, due to heating and the addition of water.

The upper row images of Figure 17 (A, B, D) contained 10 g moisture/100 g sample, but were fractured at different temperatures. The SEM images indicate that an increase in temperature led to more disentanglement between protein and starch. However, because the single pea breakage experiments are not completely representative for fracture behaviour during impact or jet milling, further investigation is required to find out whether the increased disentanglement is effective under more realistic conditions. Moreover, it should be evaluated if the rubbery state of the proteins does not affect the overall size reduction process, which again could have a negative effect because the peas cannot be milled to sufficiently small particles for subsequent separation.

Figure 17 Microstructure of broken peas: (A) 20ºC, 10%, (B) 50ºC, 10% moisture, (C) 20ºC, 15% moisture, (D) 90ºC, 10 g moisture/100 g sample, (E) 50ºC, 15 g moisture/100 g sample. PB= Protein body, CW= Cell wall, S= Starch granule, S= disentangled starch granule.*



3.5 Conclusions

Mechanistic insight in fracture behaviour of peas supports design of optimal milling and dry fractionation procedures for production of pea protein concentrates. To provide a basis for understanding fracture behaviour leading to complete disentanglement, thermomechanical morphology of peas was investigated as a function of moisture content and temperature. A state diagram was constructed for pea protein and starch based on DSC results and confirmed by Thermal Mechanical Compression Tests. The T_g curves were obtained by describing experimental results with the Gordon-Taylor equation. Three regions existed in the relevant area in the state diagram of peas: glassy protein and starch, rubbery protein and glassy starch and, rubbery protein and starch. Based on this insight, peas were analysed for their fracture behaviour in the three

regions. Glassy peas were observed to fracture at a smaller critical compression distance compared to rubbery peas. Elastic compression behaviour was observed for peas in the rubbery state. The latter is expected to have negative impact on the size reduction process. SEM analysis further indicated that the fracture plane of glassy peas is smoother, while in the fracture plane of rubbery peas separate starch granules can be seen, indicating that more disentanglement of protein and starch has taken place. It can be concluded that more energy is needed to break rubbery peas, however also more disentanglement upon fracture takes place. Further investigations on milling behaviour of peas at increased temperature and/or moisture content should reveal whether peas with only protein in the rubbery state could provide more pure protein fractions or that the overall size reduction of this material is insufficient for adequate further dry fractionation.

3.6 Acknowledgements

The authors thank The PEAS Foundation and ISPT for financial support through the Intensified Protein Structuring project. We also thank Desiree Nieuwenhuijse for her support by performing experimental work and Herman de Beukelaer for his help with interpretation of the DSC results.

4 Functional analysis of mildly refined fractions from yellow pea

This chapter has been published as: Pelgrom, P.J.M., Boom, R.M., Schutyser, M.A.I. (2015) Functional analysis of mildly refined fractions from yellow pea. Food Hydrocolloids, 44, 12-22.

4.1 Abstract

Dry fractionation offers an attractive route to sustainably produce protein-enriched plant-based ingredients. For example, fine milling of peas followed by air classification separates starch granules from the protein matrix. Unlike conventional wet isolates, dry-enriched pea fractions consist of a mixture of protein, starch and fibre, but have the advantage that protein retains its native state. In this context, dry-enriched pea ingredients were assessed for their functionality in terms of gelatinization and phase behaviour. After suspension in water, starch, protein and fibre separated into distinctive layers. The top layers were concentrated by ultrafiltration into a native protein-rich concentrate with a purity of 77g protein/100g dry matter and a protein yield of 63%. Upon heat-induced gelatinization, gel firmness was mainly increased by the presence of starch, while the presence of dispersed components (i.e. protein and/or fibre patches) in the gel weakened its structure. The heating and cooling rates influenced the firmness of the gel prepared from flour. The fine fraction could be gelled by protein crosslinking using transglutaminase. The increased protein gel strength in the presence of dispersed fibre and starch was explained by their water absorption leading to concentration of the protein phase. In conclusion, all pea fractions could be used to prepare firm gels, despite their different compositions, which supports recent insight that development of novel food ingredients should focus on functionality rather than on molecular purity. Finally, the combination of dry and aqueous phase separation is proposed as a more sustainable route compared to conventional wet extraction processes.

4.2 Introduction

The replacement of animal protein by plant-based alternatives contributes to a more sustainable future food supply (Aiking, 2011). Not only the growing world population, but also the increasing prosperity in large parts of the world have an enormous impact on the global meat consumption. Because of the poor conversion efficiency, i.e. one kg of animal protein can only be obtained by feeding six kgs of plant proteins (Pimentel and Pimentel, 2003), meat production represents a severe burden on the available arable land, water and fossil fuels. Current generation plant-based meat analogues are produced from protein-rich ingredients obtained by wet extraction, which yields a relatively pure protein isolate, but at the expense of high water and energy consumption and loss of native protein functionality (due to dissolution, precipitation and drying). We explore the use of dry fractionation via milling and air classification as a more sustainable extraction route that demands less energy and retains native protein functionality, but with the disadvantage that it produces less pure protein fractions. Schutyser and van der Goot (2011) estimated that dry fractionation requires about 4.2–18 kJ/kg protein, while wet fractionation requires approximately 18 MJ/kg protein.

Dry fractionation of peas involves fine milling during which starch granules are liberated from a protein matrix that breaks in small fragments. During subsequent air classification, the protein fragments are separated from the starch granules on the basis of their size. A pea protein concentrate (fine fraction) is obtained with 50-55 g protein/100 g dry matter and a pea starch concentrate (coarse fraction) is obtained with ~ 67 g starch/100 g dry matter (Pelgrom, Schutyser et al., 2013). Both fine and coarse fractions can be characterised by their specific ratio between three main components: protein, starch and fibre. The application of both fine and coarse fractions may contribute to a more sustainable food production. Moreover, foods themselves usually consist of a mixture of protein, carbohydrate and fibre. Whereas there is a tendency in food industry to compose foods by blending refined ingredient isolates, many traditional foods owe their attractive properties to the presence of and interaction between different constituents in the raw material. For example,

the phase separation between gluten and starch in flour determines the texture development in bread to a large degree (Schutyser and Van der Goot, 2011). Therefore, dry but not pure fractionated ingredients that retain their native functional properties (Wright and Boulter, 1980; Sosulski, Hoover et al., 1985) have large potential for preparation of foods. Here we present the results of our investigations of the interactions between starch, protein and fibre in dry-fractionated peas during phase separation and gelatinization, which are important indicators for practical application into solid, textured protein foods (e.g. meat analogues).

Air-classified native pea protein concentrates are highly soluble in water and are therefore interesting ingredients for preparing liquid high protein foods (Pelgrom, Schutyser et al., 2013). However, for solid foods a firmer texture is required. Wet pea protein isolates have been subject to many investigations to prepare gels (O'Kane, Vereijken et al., 2005; Shand, Ya et al., 2007) and to make fibrous structures (Osen, Toelstede et al., 2014), but have also been explored for foaming (Aluko, Mofolasayo et al., 2009) or emulsifying (Karaca, Low et al., 2011). In contrast, the phase separation or gelling behaviour of air classified pea fractions has received relatively little attention. Pea protein, starch and fibre will phase separate, when suspended in water. Both enthalpic and entropic effects can explain why different biopolymers phase separate when suspended in water (Elgadir, Akanda et al., 2012). Phase separation between starch and gluten is, for example, observed when wheat flour is suspended in water (Czuchajowska and Pomeranz, 1993; Larsson and Eliasson, 1996). Air classified pea fractions form gels upon heating (Sosulski and Youngs, 1979; Swanson, 1990) and for legumes it is suggested that gelation depends on the type of proteins and on the non-protein components (Sathe and Salunkhe, 1981).

Heat-induced gelatinization has been widely investigated for mixed protein-starch systems. Protein unfolds and aggregates to form a structured matrix. A protein gel may then be formed due to non-covalent crosslinks via hydrophobic interactions, hydrogen bonds and electrostatic interactions (Totosaus, Montejano et al., 2002). Starch contributes to the gelatinization when starch granules swell

upon hydration and leak amylose upon heating. Amylose then forms a network between the starch granules (Morris, 1990).

Heating mixtures of protein and starch isolates may lead to different types of gels. For example, corn starch mixed with whey protein isolate (ratio 50/50) at a dry matter content of 30 g/100 g results in a homogeneous network of leaked amylose and aggregated whey protein isolate in which the collapsed starch granules were tightly packed (Shim and Mulvaney, 2001). A similar gel structure is obtained when lentil protein isolate and lentil starch were mixed in a ratio of starch to protein of less than one, but, at higher starch concentrations, a non-homogeneous amylose network is formed, interrupted by protein-rich domains (Joshi, Aldred et al., 2014). Besides the protein and starch ratio, the heating and the cooling rates also influence gelatinization behaviour. Slow cooling allows protein more time to arrange into a network and fast heating slows down phase separation. These two factors favour the formation of a protein network (Totosaus, Montejano et al., 2002; Nunes, Raymundo et al., 2006). Gelatinization can also be enzymatically induced by crosslinking proteins (Sun and Arntfield, 2011).

Air classification can provide fractions of varying protein, fibre and starch composition and with native protein functionality. Our study aimed to explore how pea fractions can be applied for preparation of solid structures, by analysing phase separation and gelatinization behaviour as a function of protein, fibre and starch content. Fractions were first characterised on their composition, rheological properties and phase separation when suspended in water. Subsequently, gelatinization behaviour induced by heating and/or enzymatic treatment was investigated by monitoring the gel strength and by confocal laser scanning microscopy (CLSM).

4.3 Materials and methods

4.3.1 Materials

Pre-dried yellow peas, *Pisum sativum*, were purchased from Alimex (Sint Kruis, The Netherlands). The yellow peas were specified by the supplier to contain 10-15 g water/100 g, 23 g protein/ 100 g, 62 g carbohydrate/100 g (of which 44 g

starch/100 g), 2 g oil/100 g, and 3 g ash/100 g. Pea protein isolates (NUTRALYS® F85G) and pea starch isolates (PEA STARCH N—735) were obtained from Roquette (Lestrem, France). Transglutaminase Activa® WM (mTG, Ajinomoto Inc., Tokyo, Japan), with an activity of 100 units/g, was used to crosslink proteins. All experiments were done in duplicate.

4.3.2 Material preparation

Peas were pre-milled into grits with a pin mill at room temperature (LV 15M, Condux-Werk, Wolfgang bei Hanau, Germany). Subsequently, the grits were milled into pea flour using a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany). The mill contains an internal rotating classifier wheel that allows the passage of fine particles while coarse particles are retained in the chamber. The classifier wheel speed was set at 4000 rpm, the air flow at 52 m^3/h, the impact mill speed at 8000 rpm, and the feed rate at 2 rpm (circa 0.75 kg/h). A thermometer inside the mill indicated that the temperature in the mill was between 16 and 34 ºC due to varying ambient air temperatures.

A fine and a coarse fraction were made by air classifying the flour in an ATP50 classifier (Hosokawa-Alpine, Augsburg, Germany). The air flow was fixed at 52 m^3/h, the classifier wheel speed at 6000 rpm, and the feed rate at 20 rpm (circa 1 kg/h) (Pelgrom, Schutyser et al., 2013).

4.3.3 Phase separation

The pea flour, and the coarse and fine fraction were further fractionated by aqueous phase separation. Solutions of 20 g/100 g were stirred for 30 min at room temperature and were centrifuged at 4500 rpm for 30 min. This set of parameters yielded clear phase separated layers based on visual observation. The layers were separated manually. The upper two layers of the fine fraction, with a dry matter content of 5 g /100 g sample, were concentrated in a stirred Amicon ultrafiltration cell (Millipore Corporation, Billerica, MA, USA) with a 5 kDa regenerated cellulose membrane (Millipore Corporation, Billerica, MA, USA). Batch filtration was carried out at a pressure of 380 kPa until a final solids concentration of 30 g/100 g.

4.3.4 Compositional analyses

The dry matter content was determined by drying 1 gram of sample overnight in an oven at 105ºC (Pelgrom, Schutyser et al., 2013).

The protein content was obtained by Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, Interscience, Breda, The Netherlands). A conversion factor of 6.25 for pea protein was used.

The protein composition was analysed by a non-reducing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using a Bio-Rad Mini-Protean 3 cell (BioRad Laboratories, Herculas, California, USA). Samples were prepared by mixing 100 µL of sample solution (1 g protein/100 ml) with 200 µL of sample buffer solution. 15 µL of each sample and a broad range marker were separated on a 12% Tris-HCl SDS-ready gel (Bio-Rad Laboratories, Hercules, USA). The gel ran at 200 Volt for 45 min. Afterwards it was rinsed with MilliQ water and stained with Biosafe Coomassie Staining Bio-Rad Laboratories, Hercules, USA).

The total starch content was determined using a Total Starch Amyloglucosidase/ α-Amylase Assay Kit (Megazyme International Ireland Ltd, Bray, Ireland).

Particle size distributions were analysed by laser diffraction using a Mastersizer (Malvern Instruments Ltd. 2000, Worcestershire, UK). The refractive index used was 0 for particles and 1.33 for water. The absorption of particles was set to 0.

The total ash content was determined with AACC official method 08-01 (AACC, 1983)

The oil content was determined with a fully automated Büchi extraction system B-811 LSV (Büchi Labortechnik AG, Flawil, Switzerland). The oil extraction was performed with petroleum ether (boiling range 40-60ºC) in Standard Soxhlet mode for 3 h with a sample-to-solvent ratio of 1:6.

4.3.5 Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) measurements were performed using a Diamond DSC (PerkinElmer, Shelton, USA). The protein denaturation temperature and the starch gelatinization temperature were measured for pea flour, pea protein concentrate, pea starch concentrate, pea protein isolates and pea starch isolates at a moisture content of 80 g water/100 g. About 40–45 mg of sample suspension was weighed in a DSC stainless steel pan. The DSC analyser was calibrated using indium, and an empty stainless steel pan was used as a reference. During the measurement, nitrogen was used as carrier gas. Samples were heated from 20ºC to 120ºC at a rate of 10ºC/min. Each sample was prepared in duplicate and each of these duplicates was measured twice. Measurements were analysed using Start Pyris Software (PerkinElmer, Shelton, USA).

4.3.6 Nitrogen solubility index

The percentage of soluble protein was determined in a nitrogen solubility test. The samples were suspended in demineralised water at a concentration of 1 g/100 ml. Measurements were done at the natural pH of the dispersed fractions: 6.4 ± 0.1 for flour and air classified fraction and 7.7 ± 0.0 for protein isolate. The suspensions were shaken at room temperature for 1 hour and then centrifuged (3000g, 20ºC for 15 minutes) (Berghout, Boom et al., 2014a). The pellets were dried and analysed for protein content. The nitrogen solubility index (NSI) was calculated as:

$$\text{NSI}\ (\%) = \left(1 - \frac{\text{insoluble protein}\ (g)}{\text{total protein}\ (g)}\right) \cdot 100\%$$

4.3.7 Viscosity measurements

The viscosity was obtained during a shear rate sweep test. For this, the samples were suspended in tap water at 30 g sample/100 g and kept overnight before measuring in a Paar Physica MCR 301 (Anton Paar GmbH, Graz, Austria) stress-controlled rheometer, equipped with the serrated plate/plate geometry (diameter 25 mm – gap 1 mm) and a solvent trap. The strain was logarithmically increased from 1% to 100% after resting the samples for 1

minute to allow relaxation of the stresses induced during sample loading. The tests were carried out at a temperature of 25°C (Pelgrom, Berghout et al.).

4.3.8 Rapid Visco Analyser

The pasting properties were determined with a standard pasting method with a Rapid Visco-Analyzer-4 (RVA) (Newport Scientific Pvt. Ltd., Warriewood, Australia). The samples were suspended in demineralised water to a total water content of 14 g/100 g. The profile was hold at 50°C for 1 min, ramp to 95°C in 3 min 42 sec, hold at 95°C for 2.5 min, cool back to 50°C in 3 min 42 sec, and hold at 50°C for 2 min. The stirring speed was 960 rpm in the first 10 seconds, followed by stirring at 160 rpm during the rest of the experiment. The results were analysed with Thermocline software (Newport Scientific Pvt. Ltd., Warriewood, Australia).

4.3.9 Gelatinization

Gels induced by heat were made of the samples in a two-step procedure to avoid phase separated gels. Suspensions of 30 g/100 g were made and shaken for 30 minutes in a Multi Reax mixer (Heidolph Instruments, Schwaback, Germany). The suspensions and a magnetic stirring rod were poured in cylindrical teflon tubes (diameter 20 mm, length 100 mm) of which both ends could be removed. The tubes were stirred and heated at 60°C for 1 hour in a dry stirring bath (2mag, Munchen, Germany). The increased viscosity hindered phase separation during the gelatinization at 90°C for 30 minutes in a water bath. The samples were cooled down to room temperature in 2 hours and slightly frozen for 1h at -18°C. The freezing was needed to remove the gel from the tube using a cylindrical plunger. While the freezing might affect the gel structure, we did not observe any syneresis, and therefore we assumed that the influence was not large. The gel was cut with a wire cutter into sections 20-mm long and defrosted.

The starch and protein content of the gel was calculated as gram of starch or protein based on wet weight in 30 g starting sample divided by the total mass of the gel. Based on these numbers, gels were made containing similar percentage

of starch or protein from starch and protein isolates. These gels contained less dry matter.

The heating and cooling rate of gels made from 30 g sample was varied. The heating rate of 6ºC/min was reduced by heating the gels in a water bath of which the temperature increased in 4h from 60ºC to 90ºC (0.13 ºC/min). The cooling rate was reduced by cooling the samples in a programmed water bath from 90ºC to 25ºC in 4h (0.27 ºC/min).

Gels with addition of transglutaminase were prepared from a 30 g/100 g suspension which was shaken for 30 minutes in a Multi Reax mixer (Heidolph Instruments, Schwaback, Germany) and a 20 g/100 g solution of transglutaminase was mixed in with an enzyme to protein ratio of 1:17.3. The mixture was transferred to cylindrical teflon tubes, which were incubated at 50ºC for 35 min in a water bath. The samples were then cooled for 1 hour and slightly frozen. Finally, the gel was cut with a wire cutter into sections 20-mm long and defrosted.

The compression strength of the gels was evaluated by a Texture Analyser (Instron-5564Series-Table-Model- Systems-Twin-column-design, Canton, USA) equipped with a 100 N or a 2000 N load cell. A 15.24 cm diameter compression plate was positioned at the base of the system and a 5.08 cm diameter compression plate was positioned at the crosshead. The sample was placed in between and compressed at a speed of 1 mm/s till a compression of 70% of the unloaded specimen height. The force-deformation curve was recorded with the Bluehill 2 Texture Profile Analysis software (Instron, Norwood, USA).

4.3.10 CLSM

Gel samples were fixated overnight in 1% glutaraldehyde, 2% paraformaldehyde, 1mM DTT and 50mM PIPES. After washing with water, the gels were mounted on stubs and transferred to a cryomicrotome (Micron CR50-H, ADAMAS-instrumenten, Rhenen, The Netherlands). Slices of 20 µm were cut at a temperature of -20ºC. The slices were stained immediately with a mixture of 0.02 mg/ml Rhodamine B, 0.01 mg/ml fluorescein isothiocyanate (FITC) and

0.1 mg/ml Calcofluor White (CFW) to stain protein, components in the aqueous phase that were not stained by Rhodamine B or CFW and cellulose respectively. The microstructure was observed using a Zeiss LSM 510-META 18 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany), which consisted of an Axiovert 200M. A 20x Plan-Neofluar/0.5 lens was chosen to take the images. CFW was exited with an Argon diode laser at the wavelength of 514 nm. FITC and Rhodamine B were exited with an Argon laser at the wavelength of 488 and 543 respectively. From each sample, images with a dimension of 167 × 167 µm were taken at different locations. The images obtained were captured and further analysed using digital image processing software (ZEN 2008 software, Carl Zeiss, Oberkochen, Germany).

4.3.11 Statistical analysis

Student's *t*-tests were performed to evaluate the differences between fractions. Differences between means were considered to be significantly different when P was smaller than 0.05.

4.4 Results and discussion

Five pea fractions that differed in composition were investigated in this study: pea flour, the coarse and fine pea fractions obtained by air classification and commercial pea starch and pea protein isolate. It may be expected that both the composition and the processing history have large influence on functional behaviour of the pea fractions. All fractions were first characterised based on their composition, rheological properties and phase separation behaviour when suspended in water. Finally, the gelatinization behaviour induced upon heat and/or enzymatic treatment was investigated by monitoring gel strength and related to composition by carrying out confocal laser scanning microscopy (CLSM).

4.4.1 Compositional analysis

Yellow pea flour contains starch, protein, oil, ash and other components, which we assume to consist of fibre primarily (Table 5). The starch-enriched fraction (the coarse fraction) and protein-enriched fraction (the fine fraction) were

obtained by dry fractionation. The composition of both fractions was similar to those reported previously (Pelgrom, Vissers et al., 2013): the fine fraction was enriched in protein, fibre, oil and ash, while the coarse fraction was depleted of these components, which is also in line with values reported by others (Tyler, Youngs et al., 1981; Wright, Bumstead et al., 1984). It is expected that the differences in oil, ash and dietary fibre content may influence gelatinization properties.

Moreover, the functional behaviour of the fractions is expected to be related to the starch gelatinization temperature and the protein denaturation temperature. These temperatures were thus determined for flour, fine fraction, coarse fraction and both commercial pea protein and starch isolates by differential scanning calorimetry. The flour, coarse fraction and starch isolate all showed gelatinization temperature between 66.3 and 71.8°C (Table 5), in agreement with values reported for pea starch: 66.9 - 67.4°C (Hoover and Ratnayake, 2002), 66.8 - 67.5°C (Ratnayake, Hoover et al., 2001), 70.7°C (Li and Yeh, 2001), and 68.4-69.9°C (Simsek, Tulbek et al., 2009). The protein in the fine fraction denatured at a temperature of 88.2°C, which corresponds to the denaturation temperature (~ 88°C) of both the legumin and the vicilin protein (Shand, Ya et al., 2007). The measured values were also in agreement with the denaturation temperatures of 91°C and 86°C measured for pea isolates and air-classified pea protein, respectively (Murray, Arntfield et al., 1985; Sosulski, Hoover et al., 1985). The denaturation enthalpy for the pea isolate was 0.1 ± 0.0 J/g protein compared to 1.3 ± 0.1 J/g protein for the fine fraction, which indicates that a substantial part of the proteins in the pea isolate denatured during isolation.

Table 5 Composition, nitrogen solubility index (NSI), gelatinisation temperature of starch and denaturation temperature of protein of the five investigated pea fractions ± absolute deviation (n=2).

Sample	Starch content (g/100 g dry matter)	Protein content (g/100 g dry matter)	Fibre* (g/100 g dry matter)	Oil content (g/100 g dry matter)
Starch isolate	84.3 ± 0.7	nd	15.7 ± 0.7	0.5 ± 0.2
Coarse	67.2 ± 1.6	9.5 ± 0.0	23.3 ± 1.6	1.3 ± 0.3
Flour	47.6 ± 1.0	22.4 ± 0.8	30.0 ± 1.3	1.9 ± 0.3
Fine	1.7 ± 0.0	49.7 ± 0.2	48.6 ± 0.2	3.8 ± 0.2
Protein isolate	nd	83.5 ± 0.2	16.5 ± 0.2	0.9 ± 0.3

*nd: not detected, *included minor additional components, i.e. 2 g/100 g dry matter fat and 3 g/100 g dry matter ash.*

Table 5 continued.

Sample	Ash content (g/100 g dry matter)	NSI (%)	$T_{gelatinization}$ (°C)	$T_{denaturation}$ (°C)
Starch isolate	0.1 ± 0.0	nd	71.8 ± 0.8	nd
Coarse	2.3 ± 0.0	89.4 ± 1.7	71.0 ± 0.0	nd
Flour	5.0 ± 0.0	85.8 ± 3.3	66.3 ± 0.2	89.2 ± 0.2
Fine	9.6 ± 0.0	85.0 ± 1.9	70.3 ± 1.4	88.2 ± 1.2
Protein isolate	5.5 ± 0.0	24.8 ± 2.2	nd	87.8 ± 0.8

4.4.2 Phase separation behaviour of dry-enriched pea fractions

The dry-fractionated samples phase separated when suspended in water. This was accelerated by centrifugation. After suspension and centrifugation, four distinct layers could be distinguished (Figure 18):

- Layer 1: A liquid layer rich in soluble (Table 6). Soluble fibre is also expected to be present in this layer (Czuchajowska and Pomeranz, 1993).

- Layer 2: Has the texture of a soft gel. For all dry ingredient fractions, this layer contains more than 60 g protein/100 g dry matter. The protein content and the yield of this layer increases with the initial protein content.

- Layer 3: A firm layer. Next to some protein, tailing starch and insoluble fibre are expected to be present (Czuchajowska and Pomeranz, 1993; Czuchajowska, Otto et al., 1998; Sayaslan, Seib et al., 2012). The yield of this layer increased with increasing fibre content.

- Layer 4: This layer has a white colour and is solid. The particle size of the starch isolate, coarse fraction and flour is 24.8 ± 0.6 µm, which indicates that this layer is rich in starch (Czuchajowska and Pomeranz, 1993; Peighambardoust, Van der Goot et al., 2005; Sayaslan, Seib et al., 2012).

The protein composition of the various layers was investigated using SDS-page, since it was expected that soluble and insoluble protein might distribute differently across layers. However, this was not confirmed, which may be explained by the high solubility of pea protein (Table 5). If all proteins completely dissolve in the water then similar proteins will be found in all phases.

The first two protein-rich layers of the fine fraction, with a combined protein content of 67.9 ± 0.1 g protein/100 g dry matter, were harvested and concentrated by ultrafiltration in an Amicon cell. The obtained solids content was 30 g/100 g with a protein percentage of 77.4 g/100 g dry matter. The increase in protein content compared to the protein content of the original layers may be explained by permeation of low molecular weight carbohydrates, like sugars and oligosaccharides. As no pH nor temperature shifts were applied, it was expected that the protein in the concentrate were still native. The protein yield of the concentrate was 82.3 g/100 g compared to the total amount of protein present the fine fraction (Table 7), and 63 g/100 g compared to the total amount of protein present in the original flour before air classification.

Conventional procedures usually operate at much lower protein concentrations (hence, use more water) and employ ultrafiltration in combination with an alkaline pH shift to 9 before filtration. The final protein content obtained is

typically between 80 and 85 g/100 g with a protein yield of 55 to 65 g/100 g (Fredrikson, Biot et al., 2001; Makri, Papalamprou et al., 2005; Boye, Aksay et al., 2010; Mondor, Tuyishime et al., 2012). Thus, while the protein yield is high and the conditions are milder, the purity is only a bit lower for this new combination of dry fractionation and aqueous phase separation compared to conventional extraction (Mondor, Tuyishime et al., 2012). Another specific advantage of first using dry fractionation is that protein-enriched flour is produced before a wet separation step, which implies that only part of the raw material has to be subjected to the wet step. This increases the efficiency of the wet separation and reduces its capital costs (smaller membrane area required). A comparison of water use and energy needed for drying was made between a conventional procedure and the phase separation process. Assuming that the conventional process starts with flour with a protein content of 21 g/100 g dry matter and extracts proteins at a dry matter/water ratio of 1:10 (Fredrikson, Biot et al., 2001), 85 kg water/kg protein isolate is needed for a conventional procedure compared to 15 kg water/kg protein isolate in the phase separation process. Conventional procedures obtain a protein isolate juice with a dry matter content of 7 g/100 g sample before drying (Fredrikson, Biot et al., 2001), while the phase separation process obtains protein isolates with a dry matter content of 30 g/100 g sample. Taking an energy use per kg evaporated water of 4.8 MJ/kg (Schutyser and Van der Goot, 2011), the conventional process uses 63 MJ/kg protein isolate, while the phase separation process uses 11 MJ/kg protein isolate.

Table 6 Protein content of phase separated pea fractions ± absolute deviation (n=2).

Sample	Protein content layer 1 (g/100 g dry matter)	Protein content layer 2 (g/100 g dry matter)	Protein content layer 3 (g/100 g dry matter)	Protein content layer 4 (g/100 g dry matter)
Coarse	42.5 ± 0.8	61.1 ± 0.6	14.0 ± 0.2	1.4 ± 0.1
Flour	55.3 ± 0.4	65.9 ± 0.3	18.7 ± 0.3	2.2 ± 0.3
Fine	68.6 ± 0.6	67.4 ± 2.7	27.0 ± 0.2	8.9 ± 2.6

Table 7 Protein yield of phase separated pea fractions ± absolute deviation (n=2).

Sample	Protein yield layer 1 (g/100 g)	Protein yield layer 2 (g/100 g)	Protein yield layer 3 (g/100 g)	Protein yield layer 4 (g/100 g)
Coarse	62.7 ± 1.4	16.2 ± 2.9	11.9 ± 1.0	9.2 ± 0.9
Flour	59.8 ± 0.7	20.8 ± 0.5	15.3 ± 0.8	4.1 ± 0.7
Fine	66.5 ± 1.9	15.7 ± 1.0	14.9 ± 1.4	2.8 ± 0.9

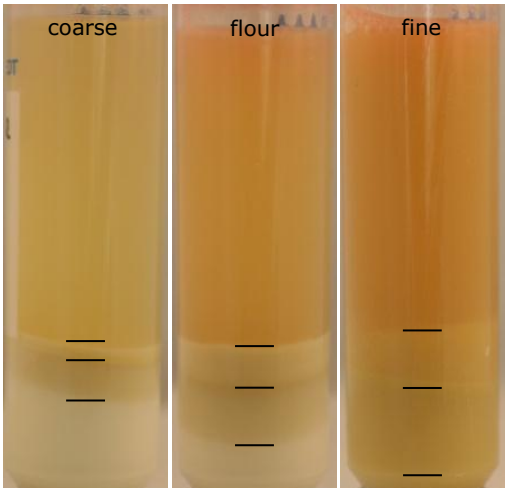


Figure 18 Phase separated coarse, flour and fine fractions. Four distinctive layers were formed (20 g sample/100 g solution).

The protein composition of the various layers was investigated using SDS-page, since it was expected that soluble and insoluble protein might distribute differently across layers. However, this was not confirmed, which may be explained by the high solubility of pea protein. If all proteins completely dissolve in the water then similar proteins will be found in all phases (Table 5). The first two protein-rich layers of the fine fraction were harvested and concentrated by ultrafiltration in an Amicon cell. The obtained solids content was 30 g/100 g with a protein percentage of 77.4 g/100 g dry matter. The increase in protein content

compared to the protein content of the original layers may be explained by permeation of soluble carbohydrates. As no pH nor temperature shifts were applied, it was expected that the protein in the concentrate were still native. The protein yield of the concentrate was 82.3 g/100 g compared to the total amount of protein present the fine fraction, and 63 g/100 g compared to the total amount of protein present in the original flour before air classification.

Conventional procedures usually operate at much lower protein concentrations (hence, use more water) and employ ultrafiltration in combination with an alkaline pH shift to 9 before filtration. The final protein content obtained is typically between 80 and 85 g/100 g with a protein yield of 55 to 65 g/100 g (Fredrikson, Biot et al., 2001; Makri, Papalamprou et al., 2005; Boye, Zare et al., 2010; Mondor, Tuyishime et al., 2012). Thus, while the protein yield is high and the conditions are milder, the purity is only a bit lower for this new combination of dry fractionation and aqueous phase separation compared to conventional extraction (Mondor, Tuyishime et al., 2012). Another specific advantage of first using dry fractionation is that protein-enriched flour is produced before a wet separation step, which implies that only part of the raw material has to be subjected to the wet step. This increases the efficiency of the wet separation and reduces its capital costs (smaller membrane area required).

4.4.3 Rheological behaviour of suspended and heated ingredient pea fractions

The rheological behaviour of aqueous pea fraction suspensions (30 g/100 g) was assessed at ambient temperature. The viscosity of the suspended dry pea fractions increased with increasing protein and fibre content (Figure 19) which may be explained by the fact that protein and fibre were partly dissolved or absorbed water (Pelgrom, Vissers et al., 2013). This means that the available free water was reduced, which led to a higher viscosity. The starch fraction had relatively little effect on the viscosity at this concentration. The viscosity of the protein isolate was high due the high water absorption capacity of denatured protein (Osen, Toelstede et al., 2014).

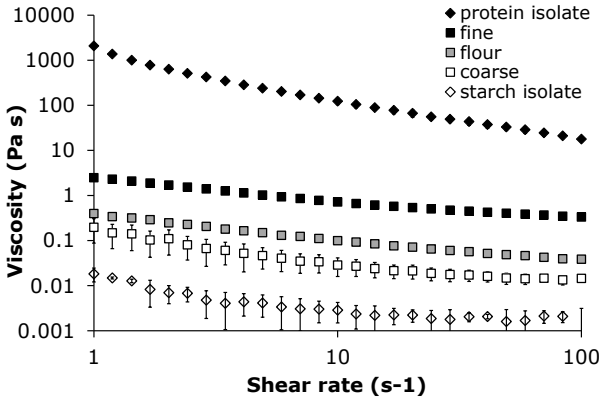


Figure 19 Viscosity as function of shear rate for the different pea fractions with specific starch to protein ratios.

Additionally, the effect of heating and cooling on the rheological properties was investigated using RVA experiments (14 g/100 g), which imposes a temperature-time profile on the material while the viscosity is followed. In Figure 20 images of the three dry fractions after RVA experiments are shown to visualise the actual rheological behaviour. The viscosity increased as the starch content increased. The pasting temperature, at which the viscosity started to increase, coincided with the gelatinization temperature of starch (66.3 - 71.8°C, Table 5) for the starch isolate and the coarse fraction (Figure 20). Starch gelatinization leads to an increase in the viscosity because the starch granules absorb water and swell. After further heating the starch granules rupture, which reduces the viscosity further (Singh, 2011). After cooling, the viscosity increased due to re-association of the starch molecules. The viscosity of the commercial protein isolate, however, decreased over time, which may be related to its high initial degree of denaturation and thus initial high water absorption and viscosity. As a result of thermal treatment and mixing, its viscosity decreased over time, which was also reported previously (Osen, Toelstede et al., 2014).

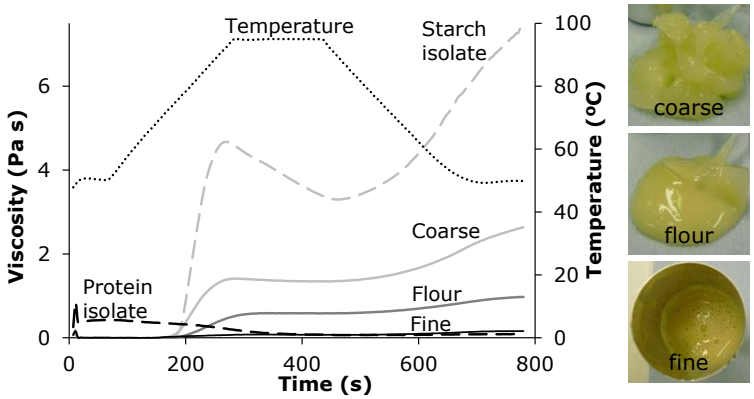


Figure 20 Effect of starch to protein ratio on the viscosity of pea fractions (n=2). Images at the right illustrate the visual appearance of the prepared suspensions of the coarse, fine and pea flour fractions.

4.4.4 Gelatinization behaviour of the pea fractions

The gelatinization behaviour of the different pea fractions upon heating was investigated. Because the protein denaturation temperature and starch gelatinization temperature are different, it was hypothesised that gelatinization behaviour would be influenced by the heating and/or cooling rate. Besides, we investigated whether enzymatic protein crosslinking could be used to enhance the gelatinization of protein-enriched pea fractions.

4.4.5 Heat-induced gelatinization behaviour

Compression tests on gels prepared from the different pea fractions showed that starch concentration has a large influence on gel strength (Figure 21), which is in agreement with RVA analysis. Gel strength increased exponentially with starch concentration, which was also observed for lentil starch–protein mixtures (Joshi, Aldred et al., 2014).

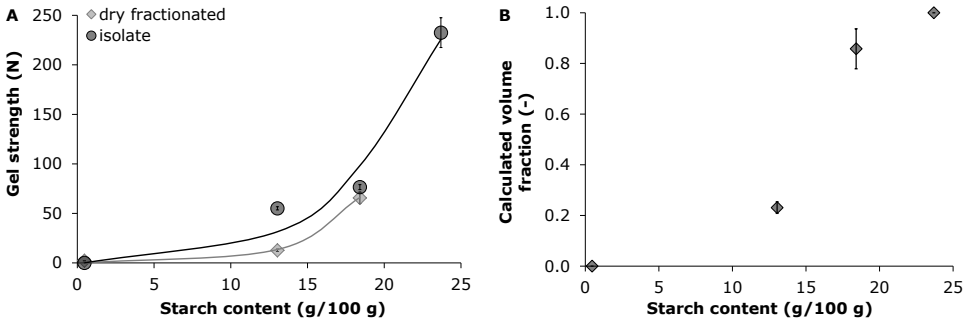


Figure 21 (A) Gel strength for dry processed fractions: 0.46 g starch/100g gel (prepared from fine fraction), 13.0 g starch/100g gel (prepared from flour), 18.4 g starch/ 100g gel (prepared from coarse fraction)) and the starch isolate at varying starch contents. The continuous lines represent the power law fitted to the experimental data ± absolute deviation (n=2). (B) Starch volume fraction of gels prepared from enriched fractions as a function of the starch content (n=2). Starch volume fraction was estimated by dividing gel strength of a dry-enriched gel by gel strength of a starch isolate gel prepared at similar starch concentration. The volume fraction of the starch isolate at 23.7 g starch/100g gel was assumed 1.0.

The power law model ($GS=k \cdot C^n$) was fitted to measured gel strength (GS) for suspended and gelled starch isolate and dry processed fractions at various starch concentrations (C) with fitting parameters k and n. This model was in analogy to what has been done before to relate storage modulus measured by small amplitude oscillatory rheology to polymer content (w/w) (Aguilera and Rojas, 1997; Gómez-Díaz and Navaza, 2003). The power law could describe the exponential increase of the gel strength with increasing starch concentration for both the gels prepared from the isolate and the dry processed fractions (Figure 21A). The gels prepared from starch isolates had a higher gel strength compared to those prepared from dry processed fractions for a given starch concentration, which may be explained by the presence of protein and fibre that form dispersed domains in the starch network weakening the network. The presence of these domains varies for the different pea fractions and affects their specific gel strength. The strength and structure of the various gels are described in the next section.

The starch isolate gel consists of swollen starch granules surrounded by a continuous phase of amylose and amylopectin that leaked from the starch

granules as described by (Eliasson and Gudmundsson, 1996). This gel has the highest strength.

The coarse fraction gel consists of aggregated protein (red) and cell wall particles (blue) surrounded by a continuous aqueous phase of connected swollen starch granules and leaked starch molecules (green) (Figure 22A). Similar observations were made by others (Joshi, Aldred et al., 2014). The gel is most probably formed by starch gelatinization around 70°C (Table 5) during which starch granules absorb water and partially disintegrate. This results in a higher viscosity leading to segregation between the viscous starch phase and the less viscous protein phase. Finally, around 88°C (Table 5) protein denatured and aggregated. Fibre and aggregated protein probably weakened the gel structure.

The flour gel structure consists of a mixture of swollen starch granules, protein-rich zones and cell wall particles (Figure 22B). Similar structures were observed earlier for corresponding starch protein ratios (Ribotta and Rosell, 2010). This gel exhibited a lower strength than the coarse fraction ($P<0.05$).

The fine fraction gel structure consists of a continuous protein phase with embedded cell wall particles and starch granules (Figure 22C). Gel strength was very poor, which may also be related to the relatively high fibre, oil and ash contents (Makri, Papalamprou et al., 2006).

The gel prepared from ultra-filtrated protein consists of a continuous protein network (Figure 22D). Gel strength (3.6 ± 0.1 N) was higher ($P<0.05$) than that of the fine fraction gel (2.2 ± 0.2 N) because protein concentration was higher and fewer dispersed components were present that could disturb the network. Still, the gel strength was relatively low compared to the fractions with higher amounts of starch. The weak protein network may be due to the formation of internal disulphide bonds in legumin protein or repulsive forces between the α-subunits of vicilin (O'Kane, Vereijken et al., 2005) that hinder protein crosslinking.

The protein isolate gel is expected to consist of a continuous network of protein. Gel strength (8.8 ± 0.7 N) was higher ($P<0.05$) than that of the fine (2.2 ± 0.2 N) or the ultra-filtrated protein fraction (3.6 ± 0.1 N). This may be explained by the higher protein content and probably also by the high water holding capacity of the denatured protein. Another unknown variable is the pea cultivar from which the protein isolate has been obtained. If the isolate is made from a different pea cultivar than the dry processed fractions this might slightly affect the gel strength (O'Kane, Vereijken et al., 2005).

Since the presence of starch has a dominant effect on gel strength, the starch volume fraction may be related to gel strength (Hongsprabhas, 2007; Fitzsimons, Mulvihill et al., 2008). For this reason we assumed that the prepared gels were rigid starch gels in which soft protein and fibre particles are dispersed (i.e. $GS_{starch} >> GS_{protein}$ and $GS_{starch} >> GS_{fibre}$). This assumption can be considered appropriate for the starch isolate and the coarse fraction and reasonable for the flour (Figure 22). Subsequently, the Takayanagi blending law for biopolymer co-gels was applied. It states that the ratio of the gel strength of the multicomponent starch gel and the starch isolate gel is representative for the volume fraction of starch in the multicomponent starch gel (Fitzsimons, Mulvihill et al., 2008). Following this law, starch volume fractions were estimated for the different gels (Figure 21B). A large difference between the starch volume fraction of the coarse fraction and the flour could be observed. This difference is in agreement with the visual observation (Figure 22) that starch is the continuous phase in the gel prepared from the coarse fraction, while a mixed gel was prepared from flour.

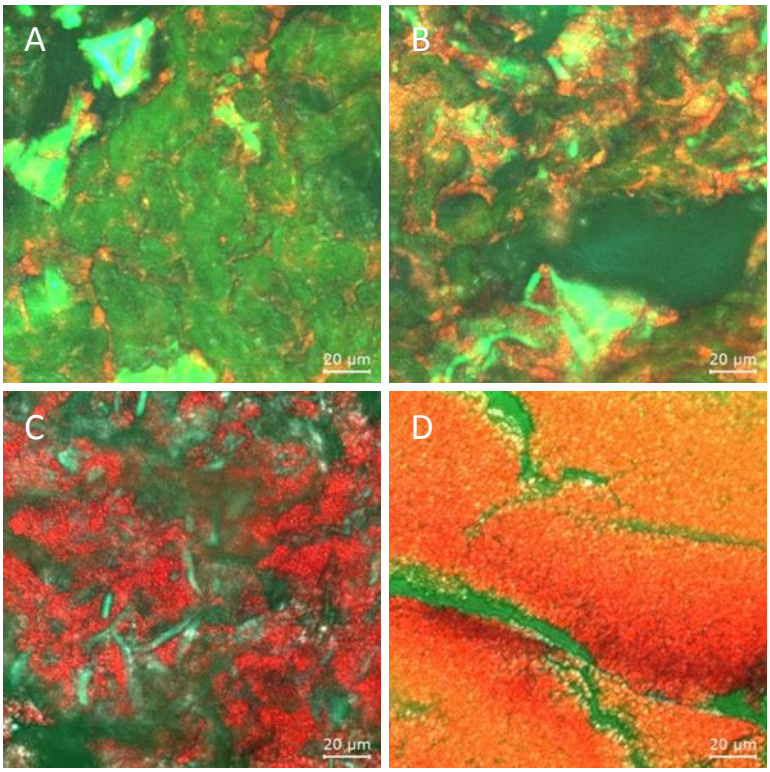


Figure 22 CLSM pictures of gels prepared from the coarse fraction (A), the flour (B), the fine fraction (C), and the ultra-filtrated protein concentrate (D). Green: aqueous phase containing starch granules in (A) and (B), red: protein, light blue: cell wall (cellulose).

4.4.6 Controlling gel strength by adjusted heating and cooling rates

The previous results showed that the gel strength of protein-rich gels was lower than that of starch-rich gels. Therefore, the heating and cooling rates during gelatinization were investigated as a strategy to obtain stronger protein-rich gels: we hypothesised that when the time between starch gelatinization and protein denaturation is extended, it may affect the final gel structure. This hypothesis was evaluated for coarse, flour and fine fractions. In addition, model pea flour was composed of protein and starch isolates at similar ratio as original pea flour to investigate whether for example protein functionality had an effect on gelatinization. Differences may indicate that the protein in the model pea flour is denatured, while that in original pea flour is still native.

Lowering the heating rate from 6°C/min to 0.1°C/min (Figure 23B) resulted in lower gel strengths for the coarse fraction and flour (Figure 23A): more extensive starch gelatinization and subsequent more phase separation between starch and protein led to a more dispersed gel. The latter was confirmed by CLSM pictures, in which more distinct patches of protein were visible in the starch matrix (Figure 24A) in comparison to the 6°C/min heating rate (Figure 24B). This lower heating rate also resulted in a drastic decrease of the gel strength for the model flour, due to the poor solubility of the pea protein isolate in addition to phase separation between starch and protein (Figure 23A). The calculated volume fractions of the starch gels were 0.33 ± 0.05 (-) and 0.26 ± 0.05 (-) for the flour gels prepared at heating rates of 0.1 and 6°C/min, respectively. The higher volume fraction may be explained by the competition for available water between starch and protein during gelatinization. The gels contain less water (~ 3.3 g water per g flour) than flour can maximally absorb (~ 4.8 g water per g flour) (Pelgrom, Vissers et al., 2013). At low heating rate, starch granules gelatinize for approximately four hours before the protein denaturation starts, while at a higher heating rate the starch gelatinization and protein denaturation occur almost simultaneously (Figure 23B). Therefore, four hours of gelatinization probably allowed starch to absorb more water, become more diluted and form a weaker gel. In the CLSM images it can be observed that the protein patches are stained more intensely red at low heating rates, which indicates that the protein phase is more concentrated and thus probably absorbed less water (Figure 24A and B).

The gel strength of the gels prepared from the fine fraction was not influenced by the heating rate. This may indicate that the strength of the protein network does not depend on the heating rate, which is in agreement with the findings of O'Kane, Vereijken, Gruppen, & Boekel (2005).

Lowering cooling rate from 0.7°C/min to 0.3°C/min (Figure 23B) resulted in an increase of the gel strength for the gels prepared from the fine fraction ($P<0.05$) (Figure 23A). The lower cooling rate may allow protein to form a stronger network (Renkema and van Vliet, 2002; Nunes, Raymundo et al., 2006). The gel strength of the coarse fraction did not increase with the lower cooling rate,

which suggests that no starch retrogradation occurred: the cooling may have been too fast for amylose to rearrange into a more crystalline structure.

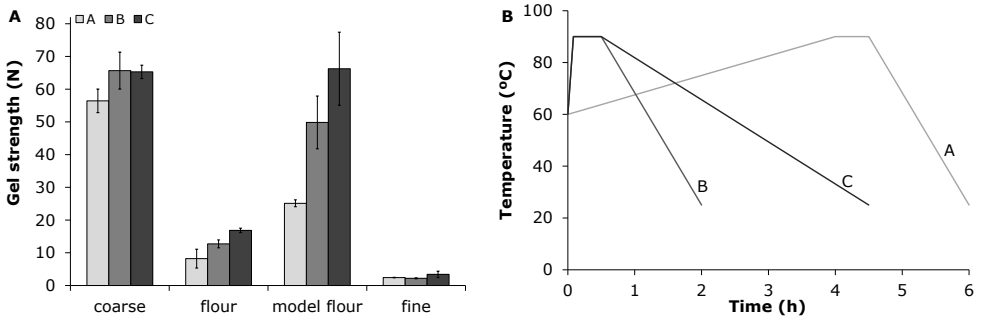


Figure 23 (A) Gel strength determined for different heating and cooling rates for gels prepared from coarse, flour, model flour and fine fraction ± absolute deviation (n=2). (B) temperature –time profiles.

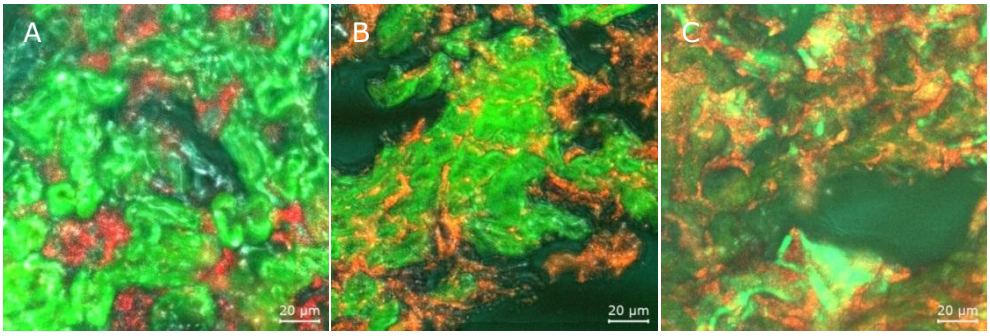


Figure 24 CLSM pictures of a gel prepared from flour with (A) a heating rate of 0.1 ºC/min and a cooling rate of 0.7 ºC/min, (B) a heating rate of 6.0 ºC/min and a cooling rate of 0.7 ºC/min, (C) a heating rate of 6.0 ºC/min and a cooling rate of 0.3 ºC/min. Green represents the aqueous phase containing starch granules, red the protein, light blue the cell wall (cellulose).

4.4.7 Controlling gel strength by enzymatic crosslinking of proteins

For the protein-rich pea fractions, we investigated whether the enzyme transglutaminase could crosslink proteins and thereby enhance gel firmness. Gelatinization experiments were carried out with the fine fraction, a model fine fraction, and protein isolate (Figure 25). The model fine fraction consisted of protein isolate diluted with water to a similar protein concentration as in the fine

fraction. Transglutaminase has not been reported previously for preparation of a gel from native pea protein concentrate, although some studies showed that protein in pea protein isolate can be crosslinked by this enzyme (Shand, Ya et al., 2008; Sun and Arntfield, 2011). Furthermore, it has been observed by others that legumin, one of the major storage proteins in pea, is a poor substrate for transglutaminase (Larre, Chiarello et al., 1993), which makes it probable that reactive groups involved during crosslinking originated from vicilin, the other major storage protein in pea.

The gels from the fine fraction and the protein isolate with added enzyme were significantly ($P<0.05$) stronger than the heat set gels (Figure 25). It appeared that, enzymatic crosslinking between glutamine residues yields stronger gels than a protein network formed during heat-induced denaturation, which is in line with previous results for pea protein isolates (Sun and Arntfield, 2011). The higher gel strength of the protein isolate may be explained by the higher protein concentration. The model fine fraction composed of diluted pea protein isolate at a similar concentration to the fine fraction yielded a lower gel strength compared to the fine fraction. Possibly, fibre in the fine fraction absorbed water enhancing the gel strength of the protein. Additionally, extensive denaturation during the wet fractionation procedure may have lowered the solubility of the protein in the isolate, making it less accessible for the enzyme. Thus using transglutaminase, a lower protein concentration can be used of fine fraction proteins to obtain gels of similar strength compared to protein isolate.

A combination of heating and enzymatic crosslinking only slightly increased the gel strength of the fine fraction ($P<0.05$). This suggests that protein denaturation in the fine fraction does not enhance the enzymatic crosslinking greatly, e.g. by exposing more reactive groups. It is hypothesized that the small increase in gel strength may be explained by more water absorption by the starch leading to an increase in protein concentration. Gel strength has been shown to exponentially increase with protein concentration, and consequently a slight increase in concentration due to starch swelling or gelatinization is reflected in the gel strength (Purwanti, Smiddy et al., 2011). The presence of

other components such as starch and fibre that absorb water may enhance the protein gel strength by a concentration effect.

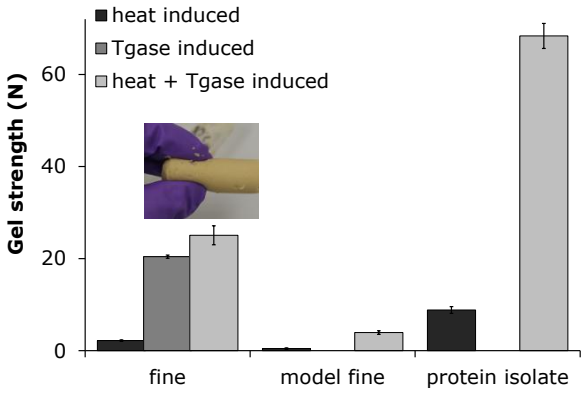


Figure 25 The influence of protein crosslinking by transglutaminase on the gel strength of fine and protein isolate gels. The image illustrates the strength and elasticity of an enzymatic and heat-induced gel prepared from the fine fraction ± absolute deviation (n=2).

4.5 Conclusions

Dry-fractionated, enriched fractions were investigated for their phase separation and gelatinization behaviour. Upon suspension in water, phase separation resulted in the formation of distinct layers that were enriched in starch, protein and/or fibre. The two top layers were highly enriched in native protein (77.4 g protein/100 g dry matter), which could be harvested and concentrated by ultrafiltration. Combined with dry fractionation this procedure was an efficient and effective extraction route with an overall protein yield of ~ 63 g/100 g, requiring 15 kg water/kg protein isolate compared to 85 kg water/kg protein isolate for conventional processes and requiring for drying 11 MJ/kg protein isolate compared to 63 MJ/kg isolate for conventional processes.

During heat-induced gelatinization, the composition strongly influenced the ultimate gel strength. The gel firmness upon heating was mainly determined by its starch content, whereas protein-rich fractions were not able to form a firm gel. By reducing the heating and cooling rates, the firmness of gels prepared

from flour could be decreased or increased, respectively. Transglutaminase could crosslink the proteins in the protein-enriched fine fraction, leading to a firm gel. The presence of other water-absorbing components in the dispersed phase increased the gel strength due to a concentration effect.

In conclusion, the combination of an initial dry fractionation step and a subsequent wet purification step, is a procedure that needs far less water and energy than completely wet routes. All pea fractions could be used as a basis to prepare firm gels despite or thanks to their different composition and the interaction between components. This functional behaviour of dry-enriched fractions gives possibilities for more sustainable food production.

4.6 Acknowledgements

The authors thank The PEAS Foundation and ISPT for financial support through the Intensified Protein Structuring project. We also thank Christa van de Peppel and Xinyu Li for their support by performing experimental work.

5 Preparation of functional lupine protein fractions by dry separation

This chapter has been published as: Pelgrom, P.J.M., Berghout, J.A.M., van der Goot, AJ., Boom, R.M., Schutyser, M.A.I. (2014). Preparation of functional lupine protein fractions by dry separation. LWT-Food Science and Technology, 59 (2), 680-688

5.1 Abstract

Lupine protein concentrate is a promising ingredient that can be obtained by a combination of milling and air classification, generally called dry fractionation. This is a more sustainable route than conventional wet extraction and delivers a protein concentrate with native functional properties. Critical is the detachment of the protein bodies from other seed components during milling. Ideally, the protein bodies are released during milling, whereas the other components remain in larger particles ($d_{0.5}$ > 40 µm) to facilitate effective air classification. Coarse milling (down to 100 µm) followed by air classification gave concentrates with protein contents between 54 - 59 g protein/100 g dry solids and yields up to 13%. The application of flowability aids (fused silica particles) during air classification doubled the yield of the protein-rich fraction. The air classified protein concentrate could provide a 2.3 times extended half-life of the foam compared to an intensively heated protein concentrate. In addition, the viscosity of the native concentrate was lower, while after (*in vitro*) digestion the amount of proteins smaller than 3 kDa was higher in native and mildly heated concentrates compared to intensively heated concentrate. These results suggest promising development of liquid-like formulations from air classified lupine protein concentrates.

5.2 Introduction

Replacement of animal protein by plant protein in foods is desirable from long-term economic and environmental points of view (Lqari, Vioque et al., 2002; Jayasena, Chih et al., 2010; Schutyser and Van der Goot, 2011). An attractive plant protein source is lupine as it can be cultivated on poor soils and in semi-tropical and moderate climates (Birk, Dovrat et al., 1990). Lupine protein has been investigated as emulsifying and foaming agent (Lqari, Vioque et al., 2002; Jayasena, Chih et al., 2010) and as an additive in bread to increase the shelf-life (Dervas, Doxastakis et al., 1999; Pollard, Stoddard et al., 2002). Further, lupine protein was explored to replace fish meal in fish feed (Robaina, Izquierdo et al., 1995; Burel, Boujard et al., 2000; Booth, Allan et al., 2001; Glencross, Evans et al., 2005; Draganovic, Goot et al., 2013). Hitherto, lupine protein is not yet applied within food industry at larger scales.

Lupine seeds contain approximately 38 g protein/100 g dry solids , mainly in the form of protein bodies (5-25 μm) (Le Gal and Rey, 1986). Other cellular components in lupine are non-starch polysaccharides (28 g/100 g dry solids), fibres (27 g/100 g dry solids), and lipids (7 g/100 g dry solids) (Evans and Cheung, 1993). Usually, lupine protein concentrates (comprising approximately 57 g protein/100 g dry solids) are prepared by wet extraction, which starts with de-hulling and coarse milling. Subsequently, the sugars and soluble non-starch polysaccharides are extracted by washing with water. A protein-rich concentrate is then obtained which is thermally dried (Dervas, Doxastakis et al., 1999). This is not only energy intensive but also affects the functionality of the protein. Dry fractionation does not require drying and thus could be an attractive alternative (Schutyser and Van der Goot, 2011).

Dry fractionation consists of milling followed by separation of the flour particles making use of differences in properties caused by compositional differences. A general method for making protein-rich concentrate is air classification, which separates on size and density differences. Small particles are carried an air flow, and thus separated from larger particles. This approach has been successfully applied to produce protein concentrates from field peas, pea beans, northern

beans, faba beans, lima beans, mung beans and lentils (Sosulski and Youngs, 1979). For those legumes, starch granules (25-40 µm) are separated from the smaller protein-rich fragments (3 µm) to yield a protein concentrate (Vose, 1978). Field peas containing 22 g protein/100 g dry solids yielded a protein concentrate containing up to 55 g protein/100 g dry solids with air classification (Pelgrom, Vissers et al., 2013). However, for lupine, protein bodies (5-25 µm) need to be separated from larger non-starch polysaccharides and fibrous fragments. The latter fragments show a wide size distribution after milling, which hinders separation by air classification (Figure 26). Hitherto, dry fractionation of lupine yielded concentrates up to 43 – 46 g protein/100 g dry solids only (Sosulski and Youngs, 1979; Carter and Hauler, 2000). The protein bodies in lupine consist of approximately 73 g protein/100 g dry solids (Plant and Moore, 1983); ideal disentanglement by milling and subsequent separation might thus deliver a protein concentrate with this protein concentration.

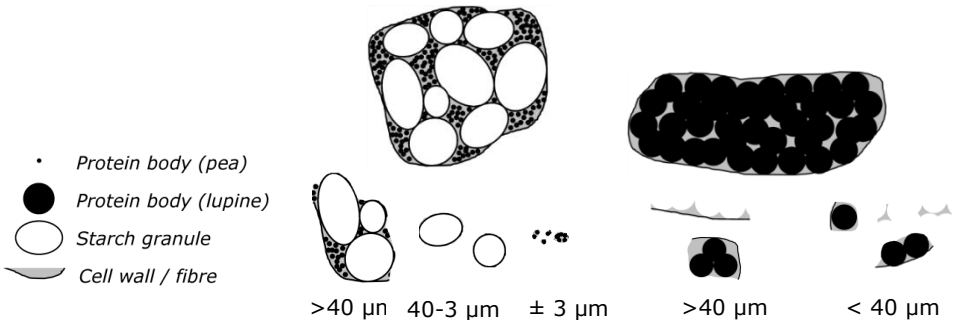


Figure 26 Schematic drawing of cells of peas (left) and lupine (right) and the fragments after milling.

Lupine contains considerable amounts of oil (7 g/100 g dry solids), which can be expected to promote the cohesion between powder particles through viscous and capillary bridging, which negatively affects the dispersion of individual powder particles in air. Besides, oil might soften the material, giving it a more ductile behaviour, and requiring more milling energy. Similar observations were reported for other lipid-rich seeds such as chick-peas, wrinkled peas and soybeans (Gueguen, 1983). Defatting of lupine prior to dry fractionation would be a straightforward approach to reduce the adhesive forces and to obtain lupine

protein concentrates with higher protein content. It has been reported that dry fractionation yielded a lupine concentrate of 61 g protein/100 g dry solids after defatting the seeds (Booth, Allan et al., 2001), though others found no significant increase in protein content (Gueguen, 1983; Dijkink and Willemsen, 2006). An alternative approach to increase the dispersibility is the use of (food grade) flowability aids, such as Aerosil. Aerosil consists of small fused silica particles of size 10 – 20 nm that adhere to the surface of the larger powder particles. Flowability aids help by increasing the distance between the powder particles, leading to a decrease in the attractive forces (Müller, Ruppel et al., 2008), an thus clump formation. Starch is used as well since it has shown to increase the dispersibility (Dijkink, 2007).

The objective of the study reported here was to prepare lupine protein concentrates by dry fractionation and characterise these for their functional behaviour. For this, the effects of milling conditions and the addition of flowability aids on the final separation efficiency with air classification were investigated. Impact milling was applied to obtain lupine flours with varying particle size distributions and dispersibilities. The dispersibility of flours with and without flowability aids was evaluated by a pressure titration method. Subsequently, lupine flours with and without flowability aids were subjected to air classification. Lupine protein concentrates obtained were analysed on their yield and protein content (amount and type). Finally, the concentrates were characterised for their functionality in terms of foam stability, viscosity and digestibility. Foam stability is an important functionality in the preparation of numerous foods (Wäsche, Müller et al., 2001; Pollard, Stoddard et al., 2002; Jayasena, Chih et al., 2010), whereas viscosity and digestibility are functional properties (in the case of lupine protein concentrate) related to preparation of liquid high-protein food formulations.

5.3 Materials and methods

5.3.1 Materials

Pre-dried lupine seeds, *Lupinus angustifolius L.*, were purchased from L.I. Frank (Twello, The Netherlands). Aerosil®200 (Azelis Netherlands B.V., Oosterhout,

The Netherlands) and potato starch (AVEBE Food, Veendam, The Netherlands) were used as flowability aids. The commercial lupine concentrate used was Fralu-con (L.I. Frank, Twello, The Netherlands). All experiments were carried out in duplicate.

5.3.2 Milling and air classification

Lupine was pre-milled into grits with a pin mill (LV 15M, Condux-Werk, Wolfgang bei Hanau, Germany). These grits had a size of 196.3 ± 7.5 µm, and were further milled with a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany). The classifier wheel was set at 1000, 2500, 4000 or 6000 rpm and the air flow was set at 80 m^3/h. Other parameters were: a feed rate of 2 rpm (circa 0.5 kg/h), an impact mill speed of 8000 rpm and a batch size of 1 kg. The milling yield was calculated as the mass of the milled flour divided by the mass of the original lupine grits.

The milled flours were air classified in an ATP50 classifier (Hosokawa-Alpine, Augsburg, Germany). The air flow was fixed at 80 m^3/h. The classifier wheel speed was set at 7000, 10000 or 13000 rpm. The feed rate was set at 15 rpm (corresponding to circa 1.0 kg/h). Per batch, 500 g of flour was air classified. Flowability aids were mixed with the flour before air classification. The yield of the fine fraction was calculated by dividing the mass of the fine fraction from the air classification, by the mass of the originally milled flour used for air classification. Similarly, the yield of the coarse fraction was calculated. The protein separation efficiency is defined as the percentage of the total flour protein recovered in the protein fraction (Tyler, Youngs et al., 1981).

5.3.3 Scanning Electron Microscope

The images of lupine flour were obtained by scanning electron microscopy (Phenom G2 Pure, Phenom-World BV, Eindhoven, the Netherlands). Carbon tabs (SPI Supplies / Structure Probe Inc., West Chester, USA) were used to fix the samples on aluminium pin mounts (SPI Supplies / Structure Probe Inc., West Chester, USA). Pre-treatment of the samples was not necessary.

5.3.4 Compositional analysis

The dry matter content was determined by drying 1 gram of sample overnight in an oven at 105ºC (Pelgrom, Schutyser et al., 2013).

The protein content was determined by Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, Interscience, Breda, The Netherlands). A conversion factor of 6.25 for lupine protein was used (Sosulski and Youngs, 1979). All protein contents reported are based on dry mass.

The particle size distribution of the samples was determined by laser diffraction using a Mastersizer 2000 equipped with a Scirocco 2000 dry dispersion unit (Malvern Instruments, Worcestershire, UK). A pressure of 400 kPa was used and the volume-based particle size distribution was calculated using the Fraunhofer theory.

The proteins in the fractions were characterised on their subunit composition by High-Performance Size-Exclusion-Chromatography (HP-SEC). The method was based on the one described by Akkermans (2008). Twenty mg of lupine flour, fine fraction and coarse fraction was dissolved in 500 µl of 0.15 mol/L Tris-HCl buffer (pH8), containing 8 mol/L guanidine chloride and 0.1M DTT to dissociate the protein subunits. After mixing for 45 minutes 215 µl of acetonitrile containing 2 ml/100 ml trifluoroacetic acid (TFA) was added. The samples were mixed for 45 minutes and centrifuged (1000 *g*, 10 min, 20°C). From the supernatant 10 µl was separated using a Phenomenex BioSep-SEC-S 4000 300x7.8mm column (Phenomenex, Torrance, USA) by an ultimate 3000 HPLC (Thermo Scientific, Sunnyvale, U.S.A.) operated with Chromeleon software (Dionex Corp., Sunnyvale, USA). The flow rate was 0.5mL/min and the absorbance was monitored at 280nm. The running buffer was a solution of 6 mol/L urea with 0.1 ml/100 ml TFA (Akkermans, Venema et al., 2008). Student t-tests were performed to evaluate the differences between the fractions. Differences between means were considered significantly different for $P<0.05$.

5.3.5 Dispersibility analysis

The dispersibility was measured by pressure titration using Mastersizer 2000 equipped with a Scirocco 2000 dry dispersion unit (Malvern Instruments, Worcestershire, UK). The particle size distribution was determined at different dispersion pressures between 20–400 kPa. It is assumed that at low air pressures the particles do not fully disperse as they remain agglomerated, while at increased dispersion pressures all individual particles will disperse resulting in a smaller particle size. The ratio between the particle size at full dispersion and the particle size at each pressure is defined as the extent of de-agglomeration (DA) (Jaffari, Forbes et al., 2013). The presence of flowability aids was corrected for (Dijkink, 2007).

5.3.6 Functionality characterization

The stability of foams stabilised with the protein concentrates was evaluated by an adapted method of Hardt (2013). Flour fractions were suspended in water at ambient temperature until 0.5 g protein/100 g and stirred for 60 minutes. Thirty mL of each suspension were poured into a glass column (12 cm in height, 6 cm in diameter) with a porous metal disk (20–30 µm pore diameter). A foam height of 12 cm was created by bubbling nitrogen using a constant supply rate of 400 ml/min into the lupine suspension. The foam height was recorded visually every minute for the first 10 minutes and after that at 15 and 20 minutes (Hardt, van der Goot et al., 2013). The experiments were performed at the natural pH value of the samples (5.85 - 5.90). The foam stabilities reported are the means of four independent measurements. Prior to the foam stability analysis, one sample of lupine protein concentrate was defatted in a fully automated Büchi extraction system B-811 LSV (Büchi Labortechnik AG, Flawil, Switzerland). The oil extraction was performed with petroleum ether (boiling range 40-60ºC) in Standard Soxhlet mode for 3 hours with a sample-to-solvent ratio of 1:6. Another sample of the same lupine protein concentrate suspension was heated in a water bath at 85ºC for 30 minutes.

The viscosity of lupine flour and lupine protein concentrate was measured with a shear rate sweep. The samples were suspended at 30 g/100 g in tap water, and a temperature treatment was applied at room temperature or at 90ºC in a water

bath for 30 minutes. The suspensions were kept overnight before measuring with a Paar Physica MCR 301 (Anton Paar GmbH, Graz, Austria) stress-controlled rheometer, equipped with a serrated plate/plate geometry (diameter 25 mm – gap 1 mm) and a solvent trap. The sample was rested for 1 minute to allow relaxation of the stresses induced during sample loading. The shear rate was increased logarithmically from 1-100 s^{-1}. The tests were performed at 25°C.

The digestibility of lupine flour and protein concentrate was determined for suspensions of 0.05 g/ml in Milli-Q water, prepared at room temperature. For the concentrate, suspensions were also heated at 60°C and at 90°C in a water bath. The suspensions were digested in simulated gastric juice at room temperature. The formulation was:: pepsin (1 g/L), mucin (1.5 g/L) , NaCl (8.8 g/L) (Sigma-Aldrich, Inc., St. Louis, MO, U.S.A.), and the pH was adjusted to 2.0 with 2 mol/L HCl (Kong and Singh, 2008). 2 mL of lupine suspension was added to 50 mL of simulated gastric juice in a glass vessel, with a water jacket connected to a water bath of 37°C (Julabo GmbH, Seelbach, Germany). The solution was stirred at 100 rpm and the vessel was sealed with Parafilm (Pechiney Plastic Packaging, Inc., IL, U.S.A.). Samples were taken after 0, 5, 10, 15, 20 and 30 minutes for further analyses.

The peptide profile after digestion was analysed by Size-Exclusion Chromatography with an Ultimate 3000 UHPLC system (Thermo Scientific, Sunnyvale, U.S.A.) equipped with a TSKgel G2000SWxl column (Tosoh Bioscience LLC, PA, U.S.A.). For analysis 0.1mL of sample was used. The running buffer consisted of 30% Acetonitrile (0.1 ml/100 ml Trifluoro Acetic Acid (TFA)) and 70% Milli-Q water. The flow rate of the running buffer was 1 mL/min and the detector was set at 214 nm. In order to standardize the molecular mass range of the chromatographic separation, the following purified proteins and amino acids were used for calibration: carbonic Anhydrase (29 kDa), α-lactalbumin (14.1 kDa), aprotinin (6.51 kDa), insulin (5.7 kDa), bacitracin (1.42 kDa) and phenylalanine(165 Da) (Sigma-Aldrich, Inc., St. Louis, MO, U.S.A.). The area under the curves was integrated and the relative area for each segment was calculated.

5.4 Results and discussion

5.4.1 Milling and air classification of lupine

Milling

Lupine seeds were milled and air classified in a two-step process to separate the protein bodies from other cellular components. The seeds were first milled into grits and then into flour using a 50 ZPS impact mill. The size of the milled flour particles depended on the classifier wheel speed. High classifier wheel speeds increased the residence time of particles in the mill, leading to more extensive milling of the particles. Increasing classifier wheel speed resulted in smaller particle sizes of approximately 280 ± 6 µm down to 10-40 µm, respectively (Figure 27). Two distinct peaks were observed in the particle size distributions at moderate velocities; the first peak at 10 µm is expected to represent a mixture of disentangled protein bodies and carbohydrate particles, and the second peak at 40 µm which represents more coarsely milled particles that are rich in carbohydrates (Figure 28).

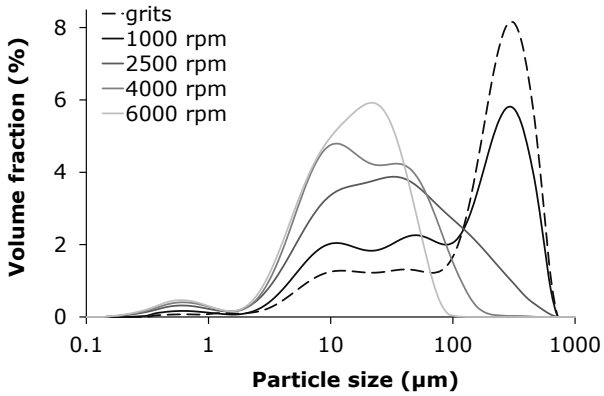


Figure 27 Particle size distributions of flours milled to grits and milled at various classifier wheel speeds. Curves represent the average of two independent measurements. The average absolute deviation was 0.72%.

Table 8 shows the average particle sizes of flours milled at various classifier wheel speeds. The highest yield (96.3 ± 0.4 g/100 g) was obtained for the most coarsely milled flour. The yield was always lower than 100%, due to the accumulation of particles on the inner walls of the mill, especially at small

batches. The yields were calculated on the basis of milling 1 kg of lupine in a pilot-scale mill. The losses (especially above 2500 rpm) increased with decreasing flour particle sizes, which may be explained by the increasing specific surface area, leading to more interactions between particles and with the walls. Van der Waals forces (Visser, 1989; Berk, 2009) are important, as a result of which larger fouling layers can be expected. Liquid bridging also contributed, because of the high oil content of lupine (Rennie, Chen et al., 1999) and because oil will be liberated from the flour upon more intensive mixing thereby enhancing the stickiness. The composition of the lost material was comparable to that of the obtained flour, which was concluded from the similar protein content of the grits and the flour milled at 6000rpm. The moisture content of the finer flours decreased somewhat during milling, due to their extended residence time in the mill and the increased surface of the particles.

Table 8 Size, yield and composition of lupine milled with an impact mill at an air flow of 80 m^3/h at various classifier wheel speeds ± absolute deviation (n=2).

Classifier wheel speed during milling (rpm)	$D_{0.5}$ (µm)	Milling yield (g/100 g)	Specific surface area (m^2/g)	Protein content (g /100 g dry solids)	Moisture content (g /100 g)
grits	196.3 ± 7.5	-	0.24 ± 0.05	39.9 ± 0.2	8.8 ± 0.0
1000	105.8 ± 31	96.3 ± 0.4	0.33 ± 0.05	37.7 ± 2.6	8.5 ± 0.2
2500	26.0 ± 2.2	71.0 ± 3.5	0.80 ± 0.04	38.5 ± 2.6	6.3 ± 0.2
4000	16.3 ± 2.0	66.3 ± 4.0	1.02 ± 0.07	45.4 ± 0.9	6.8 ± 0.5
6000	14.1 ± 0.3	68.3 ± 2.0	1.13 ± 0.04	40.3 ± 1.7	6.1 ± 0.8

Air classification

The five flours described in the previous section were air-classified at 7000 rpm into a fine and a coarse fraction (Table 9). The average size of the particles in the fine fraction was 11.7 ± 0.8 µm; the corresponding $D_{0.1}$ and $D_{0.9}$ were 3.7 ± 0.1 µm and 31.1 ± 3.0 µm, respectively, implying that isolated protein bodies with a size between 5 and 25 µm, should be in the fine fraction. Scanning

Electron Microscope imaging (Figure 28) confirmed that disentangled protein bodies were present in all flours, including the most coarsely milled flour. The protein content of the fine fraction was higher for the more coarsely milled flours; i.e. the grits and the flour milled at 1000 rpm. Upon further milling, the proportion of the disentangled protein bodies in the fine fraction increased; however, also more small particles were obtained that consisted of non-starch polysaccharides and fibres. Therefore, further size reduction led to a decrease in purity of the obtained protein concentrate (fine fraction). However, finer milling increased the overall volume of the fine fraction. This is also reflected in the increased protein separation efficiency (PSE), which is the percentage of the total flour protein recovered in the protein fraction. A higher PSE was negatively correlated with the protein content, which was observed for air classification of other crops such as wheat and pulses as well (Schutyser and Van der Goot, 2011).

Table 9 Air classification results for size, protein content and yield of lupine milled at different classifier wheel speeds ± absolute deviation (n=2). The air classification process itself was carried out at a classifier wheel speed of 7000 rpm.

Classifier wheel speed during milling (rpm)	$D_{0.5}$ fine fraction (µm)	Protein content fine fraction (g /100 g dry solids)	Protein content coarse fraction (g /100 g dry solids)	Yield fine fraction (g/100 g)	Protein separation efficiency (%)
grits	10.9 ± 0.1	52.4 ± 0.9	32.8 ± 0.5	4.5 ± 0.4	6.0 ± 0.7
1000	11.4 ± 0.6	53.7 ± 0.9	30.6 ± 0.1	12.8 ± 0.7	18.6 ± 0.8
2500	12.0 ± 0.7	49.8 ± 1.3	29.7 ± 1.1	49.8 ± 2.8	58.4 ± 4.6
4000	11.5 ± 1.1	49.3 ± 4.3	31.3 ± 0.7	58.4 ± 3.5	63.2 ± 1.7
6000	12.6 ± 0.3	46.2 ± 1.3	24.2 ± 0.1	82.4 ± 2.5	94.2 ± 0.22

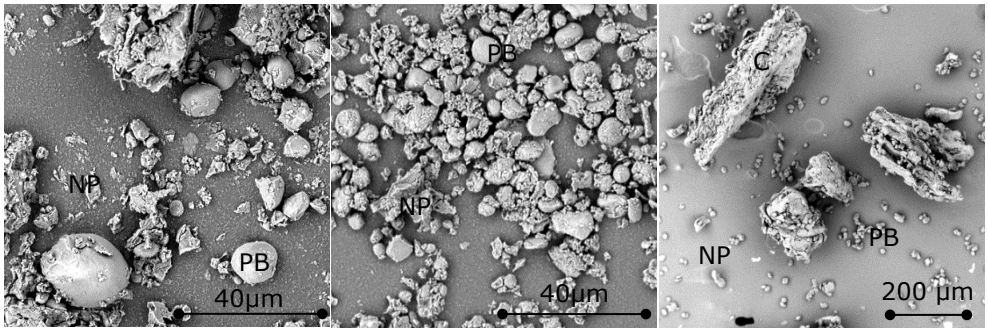


Figure 28 Scanning Electron Microscope images of lupine flour milled to grits (left), its fine fraction (middle) and its coarse fraction (right). Protein bodies (PB), pieces of non-protein cell components (NP) and clusters of cellular material (CM) can be distinguished.

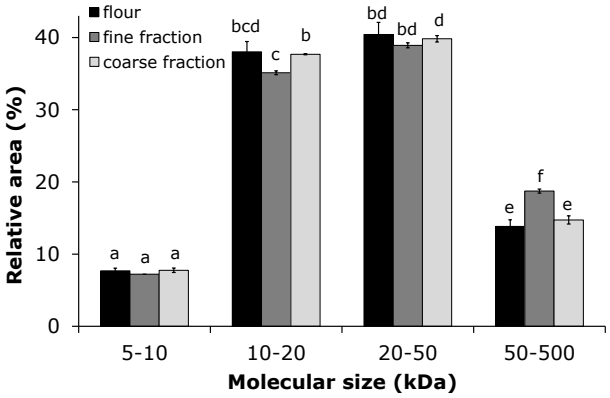


Figure 29 Molecular size distribution of the protein subunits of lupine flour, fine fraction and coarse fraction ± absolute deviation (n=2). Different letters indicate significant difference (P < 0.05).

It was hypothesised that the type of proteins in the fine and the coarse fraction are different as the protein bodies are mainly enriched in the fine fraction while the cell walls (with other proteins) are more enriched in the coarse fraction. Therefore, the subunit sizes of the proteins in the flour, the fine and in the coarse fraction were characterized by HP-SEC. It was found that the fine fraction contained more larger subunits in the 50-500 kDa size range than the flour and the coarse fraction (P = 0.027) (Figure 29). These subunits are expected to originate from β-conglutin, which is the main storage protein in the protein

bodies (Duranti, Consonni et al., 2008). The coarse fraction was richer in small subunits (5-50 kDa), which originate most probably to a large extent from albumins (Duranti, Consonni et al., 2008; Wong, Pitts et al., 2013). Albumins are a family of globular proteins and comprise enzymatic proteins, protease inhibitors, amylase inhibitors and lectins (Boye, Zare et al., 2010). Albumins facilitate cellular processes and it is therefore not likely that they are present in the protein bodies at high levels.

Table 10 Size, protein content and yield of lupine flour air classified at various classifier wheel speeds ± absolute deviation (n=2). The milling process was carried out at a classifier wheel speed of 1000 rpm.

Classifier wheel speed during classification (rpm)	$D_{0.5}$ fine fraction (µm)	Protein content fine fraction (g /100 g dry solids)	Protein content coarse fraction (g /100 g dry solids)	Yield fine fraction (g/100 g)	Yield coarse fraction (g/100 g)
7000	11.4 ± 0.6	53.7 ± 0.9	30.6 ± 0.1	12.8 ± 0.7	75.7 ± 1.0
10000	8.4 ± 0.2	58.7 ± 0.0	31.7 ± 2.3	10.1 ± 1.5	71.7 ± 1.8
13000	6.3 ± 0.0	58.9 ± 0.0	31.0 ± 4.3	6.1 ± 0.1	61.7 ± 0.4

An attempt was made to further isolate the disentangled protein bodies from non-protein cell components by air classification at increased classifier wheel speeds. Lupine flour milled at 1000 rpm was selected as starting material, because of its high protein content. As expected, particle size of the fine fraction decreased with increasing classifier wheel speeds (Table 10). The protein content of the fine fraction increased as well, suggesting that smaller particles in the flour contain more protein. Protein concentrates with higher protein content obtained were compared to those reported in literature (Sosulski and Youngs, 1979; Carter and Hauler, 2000). However, the theoretical maximum protein content of 73 g protein/100 g dry solids was not achieved. This maximum is based on the measured protein content in the protein bodies (Plant and Moore, 1983). This is explained by the presence of small non-protein fibrous particles of

similar size as the protein bodies, which cannot be removed by size-based separation.

5.4.2 The application of flowability aids during air classification of lupine

Dispersibility measurements

Particle-particle adhesion influences the dispersibility of flours in air. Lupine flour is a so-called Geldart group C powder, which means that it is difficult to disperse (Geldart, 1973). The extent of De-Agglomeration (DA), which is a measure of the dispersibility of a powder in air, was determined by pressure titration. Generally, finer milled lupine flour was found to disperse better in air at lower pressures (Figure 30), which is explained by the lower minimum fluidization velocity at smaller particle size (Geldart, 1973).

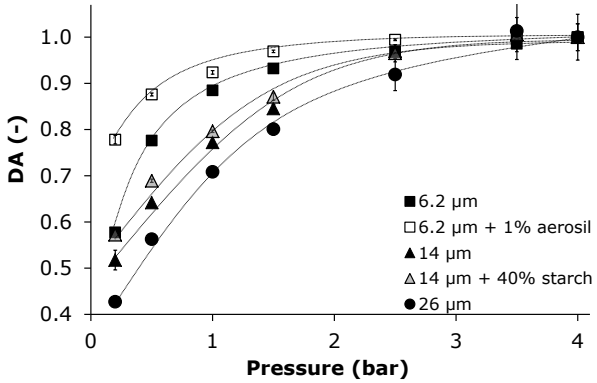


Figure 30 De-agglomeration profiles of various lupine samples ± absolute deviation (n=2). The lines are added to guide the eye.

The dispersibility of the lupine powders and thus also their air classification may be improved by addition of flowability aids (Geldart, 1973). Additions of Aerosil (12 nm) and potato starch (43.7 ± 0.4 µm) particles were evaluated. At pressures > 250 kPa, all flours dispersed well (Figure 30), but at lower pressures, Aerosil and potato starch could increase dispersibility. Most probably, the small Aerosil particles increased dispersibility by coating the surface of the lupine particles. Their presence probably increased the distance between lupine particles and reduced the van der Waals forces between particles (Villota,

Hawkes et al., 1986; Yang, Sliva et al., 2005; Müller, Ruppel et al., 2008). Potato starch granules can enhance flowability by acting as carriers for the smaller lupine particles. Indeed, it was observed that lupine particles adhered to the surface of the starch granules (Figure 31) and measured dispersibility reflected the dispersibility of potato starch alone, which was higher than that of lupine flour (Dijkink, 2007).

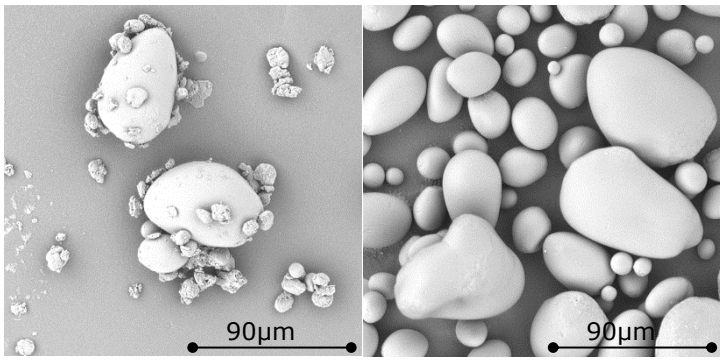


Figure 31 Scanning Electron Microscope images of lupine particles adhered to potato starch granules (left) and pure potato starch granules (right).

Air classification experiments

Aerosil and potato starch were found to enhance the lupine flour dispersibility and thus potentially improve the air classification. Addition of 1 g /100 g Aerosil improved the yield of the fine fraction (Table 11). However, the addition of flowability aids also led to a slight decrease in protein content of the fine fraction. When the dilution with Aerosil was accounted for, assuming that all Aerosil went to the fine fraction, the corrected protein content was approximately 53.9 ± 1.8 g protein/100 g dry solids, which was still less than for lupine flour without flowability aids. This may be explained by the fact that small non-protein particles in the flour also ended up in the fine fraction. Still, the addition of Aerosil improved the overall protein separation efficiency.

The addition of potato starch decreased the yield of the fine fraction. This is in line with the idea that the small lupine particles adhered to the potato starch

granules as a result of which they form larger aggregates. Thus, part of the protein bodies went into the coarse fraction.

We concluded that while the addition of flowability aids may result in a higher yield of the fine fraction, it did not lead to a fine fraction with higher purity. The protein bodies and the fibres had similar sizes and therefore an increase in dispersibility did not improve the separation. This is in contrast with earlier observations for wheat flour, in which an increase in the dispersibility led to detachment of small protein fragments from starch granules and thus better separation (Dijkink, 2007).

Table 11 Air classification results of lupine with flowability aids milled at 1000 rpm and air classified at 13000 rpm ± absolute deviation (n=2).

Sample	$D_{0.5}$ fine fraction (µm)	Protein content fine fraction (g /100 g dry solids)	Protein content coarse fraction (g /100 g dry solids)	Yield fine fraction (g/100 g)	Protein separation efficiency (%)
No flowability aid	6.3 ± 0.0	58.9 ± 0.0	31.0 ± 4.3	6.1 ± 0.1	10.0 ± 0.3
1% Aerosil	5.2 ± 0.1	49.7 ± 0.5	32.9 ± 1.2	13.9 ± 3.9	21.3 ± 5.8
40% potato starch	6.4 ± 0.2	56.2 ± 1.2	30.0 ± 0.5	4.0 ± 0.0	9.3 ± 0.0

5.4.3 Protein functionality

Lupine protein concentrates were characterised and compared to lupine flour for functional behaviour in terms of foam stability, viscosity and digestibility. Additionally, heat treatments were applied to the lupine protein concentrates to compare the functional behaviour of native and denatured protein.

Foam stability

he foam stability that could be achieved with the fine fraction (the lupine protein concentrate) was higher than that of the original lupine flour (Figure 32). The protein content of the solution was kept constant at 0.5 g protein/100 g and

therefore difference in protein content could not explain the enhanced stability. However, particles are known to destabilise foam, and since lupine flour contains more insoluble, non-starch polysaccharides and fibres, this may be the reason for the decreased foam stability (Sathe, Deshpande et al., 1982). A similar effect was observed when potato starch or Aerosil was added. Presence of more lipid droplets in the lupine flour is expected to destabilise the foam as well, as protein adsorbs at the oil-water interface, leaving less protein for the stabilisation of the foam itself (Sosulski and Youngs, 1979; Sathe, Deshpande et al., 1982; Tolstoguzov, 1997; Pollard, Stoddard et al., 2002).

To compare the non-heated, dry fractions with conventional fractions, a lupine protein concentrate solution was heated at 85ºC for 30 min. The functionality of this heated protein concentrates may be regarded similar to wet extracted protein, which is subjected to heating and drying. A decrease in foam stability was observed with the heated protein concentrate. The latter may be explained by the presence of larger protein aggregates that mostly likely fail to unfold at the water-air interface to stabilize the foam. It is noted that others found mild heating at for example 60ºC to be beneficial for foam stability (Raymundo, Empis et al., 1998). More stable foam is formed because proteins unfold, which increases hydrophobic characteristics and therefore facilitates foaming. A commercial lupine concentrate prepared from toasted lupine seeds was evaluated on foam stability as well, however no foam was formed. The latter may be explained by the application of process steps like pH changes or drying that may have affected the native properties of the wet extracted proteins. Dry fractionation could therefore be an interesting alternative to obtain protein concentrates with native properties offering still the possibility to modify functionality by moderate heating.

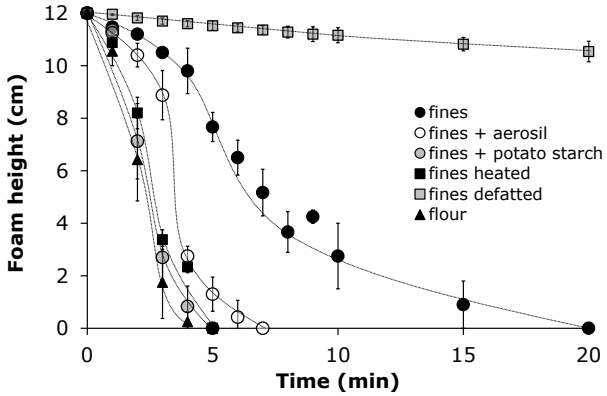


Figure 32 Foam stability various lupine samples ± absolute deviation (n=4). Lines are added to guide the eye.

Viscosity

Native lupine protein concentrate suspensions were found to exhibit lower viscosities than those of lupine flour (Figure 33). The suspension viscosity of the native protein concentrate may be compared to that of a fruit juice, while the suspension viscosity of native flour was similar to that of fruit purees at a shear rate of 100 s^{-1} (Steffe, 1996). The higher suspension viscosity of flour is probably caused by the presence of fibres (Dijkink, De Bie et al., 2008). The viscosity increased upon heating for both the flour and the concentrate, which may be explained by aggregation of proteins (Wagner, Sorgentini et al., 1992) and the increased water absorption capacity of fibres (Dijkink, De Bie et al., 2008). Both processes were more pronounced at 90ºC than at 60ºC. The latter increases the viscosity of denatured, commercial lupine flour and concentrate upon heating as well (Dijkink, De Bie et al., 2008). All samples exhibited shear thinning, which was more pronounced for the heat treated samples probably due to the relative larger influence of the fibres. The measured suspension viscosity of native lupine protein concentrate was found lower compared to viscosity values for lupine protein isolates found in literature at similar protein and dry matter contents (Xu, Mohamed et al., 2006).

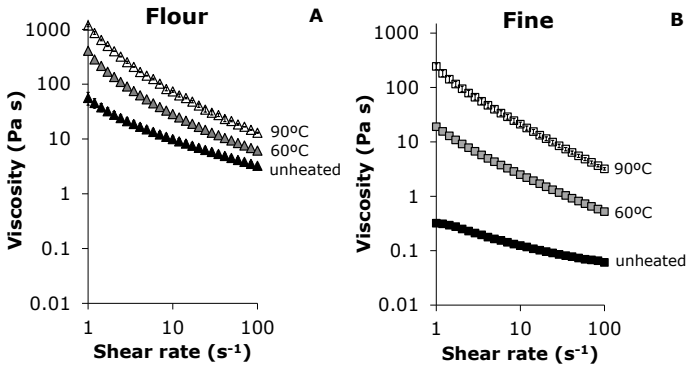


Figure 33 Viscosity as function of the shear rate for lupine flour (A) and the fine fraction (B) native (filled markers) and after a 60ºC (markers, grey fill) and 90ºC (open markers) heat treatment ± absolute deviation (n=2).

The low suspension viscosity of the native (dry fractionated) lupine protein concentrate at a protein concentration of 17.5 g protein/100 g (30 g protein / 100g dry solids) suggests potential application for protein-based beverages, in which high viscosity could adversely affect acceptability for consumers (Chew, Casey et al., 2003). Finally, the viscosity of the native protein concentrate can be tuned by selection of an optimal heat treatment.

Digestibility

Lupine flour and the fine fraction were subjected to an *in vitro* digestion test, and the peptides released into the gastric solution was analysed with HPLC analysis. Small differences in protein composition between raw materials were found. The whole flour consisted of more fragments smaller than 3kDa compared to the fine fraction, which is explained by the presence of more storage proteins in the fine fraction (Figure 34). The unheated fine fraction and the fine fraction heated at 60ºC had initially similar amount of smaller peptides, while the fine fraction heated at 90ºC had less small peptides, possibly caused by protein aggregation due to heating.

In the lupine flour, relatively less protein was digested compared to the concentrates. This may be explained by the lower protein content and reduced

accessibility of the larger particles of the lupine flour. Viscosity was probably not of influence as the system was diluted.

The final amount of peptides is higher for native and mildly heated protein concentrate compared to the intensively heated protein concentrate. Taking the raw protein concentrate as starting point, the amount of peptides smaller than 3kDa increased with 33.2 % ± 3.2 during digestion for the unheated and the concentrate heated at 60ºC. For the protein concentrate heated until 90ºC, the amount of proteins smaller than 3kDa increased only with 15.5 % ± 6.5. However, the digestion rate, reflected by the difference between final amount of fragments and initial amount of fragments, was not influenced by the heat treatment. In literature, contradictory results are presented about the effect of heating on the digestibility of legumes. Embaby (2010) found for example that more lupine protein was digested after heat treatment and Carbonaro (1997) described that the amount of digested protein in chickpea and dry bean improved upon heating, the protein digestibility of lentils was not affected and that of faba bean decreased. Parameters of influence to digestibility of legume proteins are amongst others the degree of protein denaturation, the presence of protease inhibitors, and the presence of anti-nutritional compounds like phytic acid, condensed tannins and polyphenols. Heating impairs protease inhibitors and anti-nutritional compounds, which enhances digestibility. Protein denaturation makes proteins more accessible to hydrolysis, but it can also cause protein aggregation making them again less accessible for digestion (Carbonaro, Cappelloni et al., 1997; Embaby, 2010).

Our results indicate that the unheated, dry-fractionated concentrates have a bioavailability that is at least similar to conventional, heated types, and thus are just as suited for application in nutritious protein products. At the same time, the results indicate that the size of the particles is an appropriate parameter to tune the release (with a finer powder, more peptides are released).

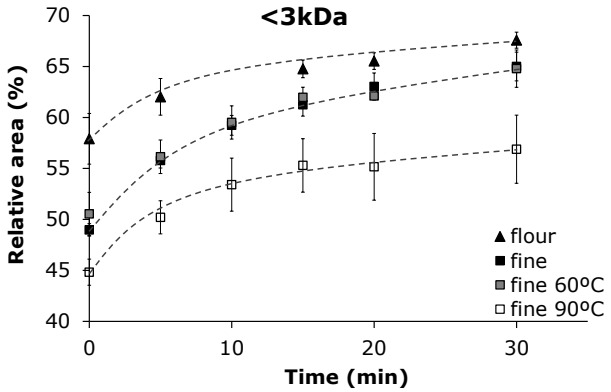


Figure 34 Digestion of lupine flour, fine fraction and the fine fraction heated at 60ºC and 90ºC during 30 minutes expressed as percentage of the area under the chromatogram ± absolute deviation (n=2). Lines are added to guide the eye.

5.5 Conclusions

Dry milling and air classification of lupine flour yielded protein concentrates with varying protein content and protein separation efficiency. Both the yield and purity were mainly determined by the degree of milling. The highest purity in the fine fraction obtained was 59 g protein/100 g dry solids. Coarse milling (down to 100 µm) was found optimal to disclose protein bodies from their matrix. Further milling led to more disclosed protein bodies, but also decreased the size of other, non-protein particles such that purity of the fine fraction was compromised. Moreover, extensive milling led to increased losses, due to build-up of a fouling layer in the mill.

Addition of Aerosil (~ 12 nm) and potato starch (~ 45 µm) powder as flowability aids successfully enhanced the dispersibility of the flour particles, but only the addition of Aerosil increased the protein separation efficiency. However, Aerosil addition also led to a decreased purity, which was explained by an increased dispersibility of other, non-protein components.

The air-classified protein concentrates with higher protein content provided more stable foams than the heat treated lupine protein concentrates. The viscosity of the native protein concentrate was found low, suggesting opportunities for the

development of high protein beverages. Finally, *in vitro* digestion of the native and a mildly heated concentrate resulted in a larger amount of small peptides compared to the more intensively heated and aggregated protein, which could indicate a nutritional benefit for the former ones.

5.6 Acknowledgements

The authors wish to thank The PEAS Foundation and ISPT for project and financial support through the Intensified Protein Structuring project. We acknowledge Anja Janssen for fruitful discussions on the digestion experiments. We also acknowledge Memet Ali Ustunel and Merel Huizing for their contribution to the experimental work.

6 Pre- and post-treatment enhance the protein enrichment from milling and air classification of legumes

This chapter has been published as: Pelgrom, P.J.M., Boom, R.M., Schutyser, M.A.I. (2015) Pre- and post-treatment enhance the protein enrichment from milling and air classification of legumes*. Journal of Food Engineering.*

6.1 Abstract

Air classification is a milder and more sustainable method to obtain protein-enriched fractions than commonly used wet fractionation. The protein content of air-classified fractions is generally lower than obtained with wet methods, therefore we applied pre- and post-treatments to increase the protein purity. A starch-rich legume, pea, and an oil-rich legume, lupine, were pre-treated by varying the moisture content, defatting, soaking or freezing cycles. Higher moisture contents and defatting of lupine increased the protein purity, but lower moisture contents increased the protein yield. Soaking and freezing cycles lowered the particle density, which impaired the separation. Electrostatic separation is based on electrostatic charging behaviour and was successfully applied to enrich air-classified fractions by separating protein and fibre into oppositely charged fractions. The results showed that pre- and post-treatments yielded protein fractions that are significantly purer than those obtained in single-step milling and air classification.

6.2 Introduction

Protein-enriched fractions can be obtained from starch-rich legumes such as pea and oilseeds such as lupine, by combined milling and air classification (Sosulski and Youngs, 1979; Tyler, Youngs et al., 1981; Schutyser and Van der Goot, 2011; Pelgrom, Vissers et al., 2013; Pelgrom, Berghout et al., 2014). The milling detaches the protein from the other cellular components. Subsequently, air classification separates the smaller protein-rich fragments from the starch granules for pea, and the protein bodies from larger cell wall rich fragments for lupine. During air classification the powder particles are fluidized and contacted with a rotating classifier wheel. Small particles below the cut size are collected via the classifier wheel in the fine fraction, depending on the gas flow and the rotation speed of the classifier wheel. Larger starch granules or fibre fragments are collected via the bottom of the classifier into the coarse fraction. During previous research we found that pea fine fractions with 55.5 ± 0.5 g protein/100 g dry matter (Tyler, Youngs et al., 1981; Wright, Bumstead et al., 1984; Pelgrom, Vissers et al., 2013) and lupine fine fractions with 58.9 ± 0.0 g protein/100 g dry matter could be obtained (Pelgrom, Berghout et al., 2014). These protein levels are lower than for protein isolates which typically have 80-85 g protein/100 g dry matter.

Higher protein content of the dry-enriched fractions could widen the perspective for applications, e.g. to better meet nutritional and/or functional ingredient requirements. Theoretically, the maximum protein content that can be achieved by air classification is the actual protein content of the protein bodies (73 g protein/100 g dry matter) (Plant and Moore, 1983), which means there is still room to increase the protein content in the fractions obtained by air classification. The improvement is expected to be achieved by adding pre-treatments or post-treatments to the separation process. A wide variety of pre-treatments has been proposed in literature to enhance the protein yield and purity. Two main types were considered in this paper, namely: weakening of the cellular structure by moisture addition or freezing and removal of specific components from the seed.

Addition of moisture has been reported to enhance disentanglement of cellular components, although its effect is species dependent (Pelgrom, Schutyser et al., 2013). A higher yield and protein content were obtained in the fine fraction for hard wheat, whereas an opposite effect was found for soft wheat, field pea and faba bean (Kent, 1965; Tyler, 1982). A disadvantage of moisture addition is that ductility of the seeds increase and therefore more energy is required for milling (Dijkink, 2002).

Another way to disrupt cellular structures may be freezing in the presence of water. During initial experiments fast freezing with liquid nitrogen of non-wetted pea and lupine seeds (11-13 g water/100 g seed) was found not effective in improving dry separation. It is hypothesized that weakening of cellular structures with ice crystals requires sufficient water and slow freezing and thawing cycles (Vertucci, 1990). Moreover, applying several freezing cycles could possibly increase the inflicted damage and thus improve the disentanglement (Fahloul, Scanlon et al., 1996). This pre-treatment has not been combined with dry fractionation and is a different approach compared to cryogenic milling. The latter treatment produces composite particles, which is not very desirable before air classification (Hemery, 2011).

The second type of pre-treatment involves the removal of specific components from the seeds before milling. For example, the hulls can be removed by air classification (Wu and Nichols, 2005) or by elusieve processing, which is a combination of air classification and sieving (Srinivasan, Hicks et al., 2010). Hulls are rich in fibre, 91 g/100 g dry matter (Ralet, Della Valle et al., 1993). Furthermore, they comprise 12-14% of the total weight of pea seeds and contain 6 g protein/100 g dry hull (Vose, 1978; Meuser, Pahne et al., 1995). Removing the hull increases the protein content of pea seeds from 22 g/100 g dry matter to 24 g/100 g dry matter. Dehulling of lupine may provide a protein content of 39 g/100 g dry matter instead of 35 g/100 g dry matter. Furthermore, lipids can be removed via extraction, which not only influences the protein content, but also the powder properties, e.g. the dispersibility of the flour (Snyder, Friedrich et al., 1984). After defatting dry fractionation can yield a lupine protein concentrate of 61 g protein/100 g dry solids (Booth, Allan et al.,

2001), although others reported no significant increase in protein content (Gueguen, 1983; Dijkink and Willemsen, 2006).

Post-treatments are another route to improve the purity and yield of dry fractionation. A post-treatment that is based on a different driving force for separation compared to particle size and density may be applied to further increase the protein content and yield (Schutyser and Van der Goot, 2011). We therefore propose to combine electrostatic separation, which relies on different tribo-electric charging properties of materials, with air classification. Electrostatic separation is not yet a common separation technique for food production, but its potential has been demonstrated for separation of wheat bran and rice flour (Noguchi, Saio et al., 1981; Hemery, Holopainen et al., 2011).

The objective of this study was to evaluate selected methods for pre- and post-treatment to increase the effectiveness of dry fractionation of pea and lupine, especially in terms of protein purity. Pea and lupine were selected as raw materials to represent starch-rich and oil-rich legumes, respectively. An initial series of lab-scale experiments was carried out to evaluate the potential of different pre-treatments. Based on protein purity increase the most promising pre-treatments were selected for more elaborate investigation: varying moisture contents, defatting and freezing cycles. Pre-treated seeds were compared to untreated seeds on: cellular structure after pre-treatment, milling behaviour and protein yield with air classification. Electrostatic separation was used as a separation method for pea and lupine flour and as a post-treatment after air classification. Finally, a reflection is provided on the boundaries of protein enrichment by air classification.

6.3 Materials and methods

6.3.1 Materials

Pre-dried yellow pea seeds, *Pisum sativum L.*, were purchased from Alimex (Sint Kruis, The Netherlands) and pre-dried lupine seeds, *Lupinus angustifolius L.*, were purchased from L.I. Frank (Twello, The Netherlands). All experiments were done in duplicate unless stated differently.

6.3.2 Preparation of enriched fractions

Pre-treatments

Blank

Untreated pea and lupine seeds were pre-milled to grits of approximately 200 µm using a Condux-Werk LV 15M mill (Condux-Werk, Wolfgang bei Hanau, Germany).

Moisture content

A decrease of the moisture content was obtained by oven drying at 50ºC for 6 days. An increase in moisture content was obtained by soaking pea or lupine overnight at 4ºC in a calculated amount of water.

Defatting

Four batches of pea and lupine grits (i.e. 4*700 g) were defatted in a soxhlet using petroleum ether (boiling range 40-60ºC) with a sample-to-solvent ratio of 1:5 for 24 h.

Soaking

Two batches of pea and lupine grits (i.e. 2*1200 g) were soaked for 1 h in 2157 g of water for peas and 2482 g of water for lupine. These amounts of water allowed complete soaking without the presence of free water at the end of the soaking time. The grits were dried in a fluidized bed (TG200 Rapid Dryer, Retsch GmbH, Haan, Germany) at an air flow of 60% (maximum: 185 m^3/h at no-load operation without material to be dried) and an air temperature of 40ºC to obtain the same moisture content as the untreated grits (13 g water/100 g pea grits and 11 g water/100 g lupine grits).

Freezing cycles

Freezing cycles were applied to whole pea and lupine seeds. The seeds were soaked overnight at 4ºC at a seed to water ratio of 1:5. After packing the seeds in plastic bags of 300 g, the seeds were frozen at -18ºC and thawed at 20ºC 3 times. A fluidized bed (TG200 Rapid Dryer, Retsch GmbH, Haan, Germany) with an air flow of 50% and an air temperature of 40ºC was used to dry the seeds to

their original moisture content of 13 g water/100 g grits for pea and 11 g water/100 g grits for lupine. The seeds were pre-milled to grits after the freezing cycles.

Lab scale milling and sieving

Milling and separation of pea and lupine at various moisture contents was done in a lab scale mill (Fritsch Pulverisette 14, Fritsch, Idar Oberstein, Germany) because in the pilot scale mill the moisture content could not be controlled due to the large air flow that is required. From the pre-treated seeds, 50 g was milled at 6000 rpm for 2 minutes with a sieve ring size of 0.2 mm for pea and 0.5 mm for lupine. The flour was separated by air jet sieving (Alpine200 LS-N, Hosokawa-Alpine, Augsburg, Germany) for 2.5 minutes at 4000 Pa on a 20 µm sieve. Each experiment started with 9.9 g of flour, which was mixed with 0.1 g fumed silica (Aerosil®200, Azelis Netherlands B.V., Oosterhout, The Netherlands) to improve the flowability. The protein separation efficiency was calculated as the amount of protein (g) in the fraction smaller than 20 µm divided by the total amount of protein (g).

Pilot scale milling and air classification

Untreated and pre-treated grits were milled into flour using a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany). This mill contains an internal rotating classifier wheel that allows the passage of fine particles while coarse particles are returned and further milled. The impact mill speed was fixed at 8000 rpm and the feed rate at 2 rpm (circa 0.75 kg/h). For pea, the classifier wheel speed was set at 3400 rpm and the air flow at 60 m^3/h. For lupine, the classifier wheel speed was set at 1200 rpm and the air flow at 80 m^3/h. The milling yield was calculated as the weight of the milled flour divided by the weight of grits. Untreated pea grits were milled at 4000 and 8000 rpm at an air flow of 52 m^3/h and untreated lupine grits were milled at 1000 rpm with an air flow of 80 m^3/h to create a wider range of samples for electrostatic separation.

Flour was separated in a coarse and fine fraction by air classification in an ATP50 classifier (Hosokawa-Alpine, Augsburg, Germany). The feed rate was fixed at 20 rpm (circa 1 kg/h). For pea, the classifier wheel speed was set at 5000 rpm and

the air flow at 52 m^3/h. For lupine, the classifier wheel speed was set at 7000 rpm and the air flow at 80 m^3/h. Untreated lupine flour was also air classified at 10000 rpm with an air flow of 80 m^3/h. The yield of the fine and the coarse fraction was calculated as weight of the fraction divided by the weight of the milled flour used for air classification.

Electrostatic separation

Untreated fine and coarse fractions from air classification, and unclassified flour were subjected to separation based on different tribo-electric charging behaviours of components. Five grams of sample were fed to a charging tube of 125 mm as described by Wang (2014). A nitrogen gas flow of 0.45 m^3/h was applied to transport the particles to the electric field created by two parallel electrodes (Figure 35). One electrode was connected to a DC power supply providing a positive voltage of 20 kV relative to the other, grounded electrode. Samples were collected from both electrodes. The protein enrichment was calculated by dividing the increase in protein content (protein content on electrode minus protein content of starting material) over the protein content of the starting material.

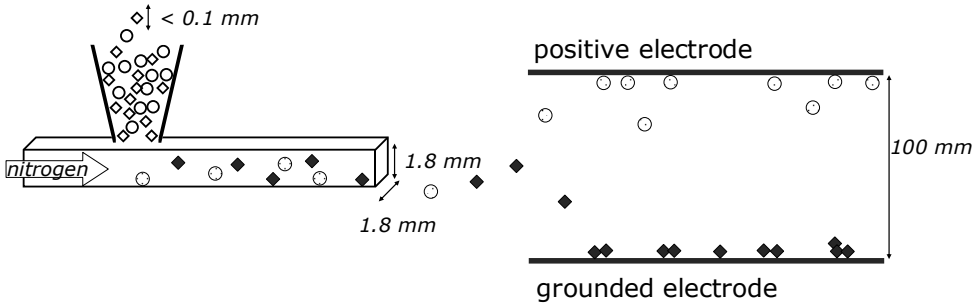


Figure 35 Schematic overview (not true to scale) of the electrostatic separation device with the electrodes drawn from the top view perspective.

6.3.3 Analyses

Compositional analyses

The dry matter content was determined by drying of 1 g of sample overnight in an oven at 105ºC.

The protein content was analysed using Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, Interscience, Breda, The Netherlands). A conversion factor of 6.25 for both pea and lupine protein was used.

The lipid content was measured in a fully automated Büchi extraction system B-811 LSV (Büchi Labortechnik AG, Flawil, Switzerland). The lipid extraction was performed with petroleum ether (boiling range 40-60ºC) in Standard Soxhlet mode for 3 h with a sample-to-solvent ratio of 1:6.

Particle size distribution

The particle size distribution of the samples was determined by laser diffraction using a Mastersizer 2000 equipped with a Scirocco 2000 dry dispersion unit (Malvern Instruments, Worcestershire, UK). A pressure of 400 kPa was used and the volume-weighted particle size distribution was calculated using the Fraunhofer theory.

Particle density

The particle density is the density of a material including air pores and was measured using a pycnometer (Ultrapyc 1200e, Quantachrome Instruments, Boynton Beach, USA), which uses nitrogen displacement to determine particle volume.

Scanning Electron Microscope

A scanning electron microscopy (Phenom G2 Pure, Phenom-World BV,Eindhoven, the Netherlands) was used to visualise the effect of the treatments. Carbon tabs (SPI Supplies / Structure Probe Inc., West Chester, USA) were used to fix the samples on aluminium pin mounts (SPI Supplies / Structure Probe Inc., West Chester, USA). Pre-treatment of the samples was not necessary due to the low acceleration voltage used (5kV).

Differential Scanning Calorimetry

Differential Scanning Calorimetry (DSC) was performed using a Diamond DSC (PerkinElmer, Shelton, USA). About 20 mg of sample was weighed in a DSC stainless steel pan. The DSC analyser was calibrated using indium, and an empty stainless steel pan was used as a reference. During the measurement, nitrogen was used as carrier gas. Samples were heated from 20ºC to 120ºC at a rate of 10ºC/min. Measurements were analysed using Start Pyris Software (PerkinElmer, Shelton, USA).

Seed hardness

The seed hardness was determined with a Texture Analyser (Instron-5564Series-Table-Model- Systems-Twin-column-design, Canton, USA) equipped with a temperature controlled sample holder (Pelgrom, Schutyser et al., 2013). The sample holder consisted of a concentric cylinder (20 mm diameter, 40 mm height) with a water chamber in the sidewalls and a solid bottom. The temperature was controlled at 20ºC by connecting the water chamber to a water bath (Julabo FP50-HE, Julabo, Seelbach, Germany). For each species 10 seeds were carefully de-hulled and split by hand. The flat-side was smoothed with glass paper. A cotyledon was, flat-side down, was compressed by a 15 mm cylindrical probe attached to a Texture Analyser equipped with a 2000 N load cell at a crosshead speed of 20 mm/min. The force-deformation curve was recorded with the Bluehill 2 Texture Profile Analysis software.

Statistical analysis

Student's-*t* tests were performed to evaluate the differences between fractions. Differences were considered to be significantly different when the p-value was smaller than 0.05.

6.4 Results and discussion

6.4.1 Pre-treatment

Influence on cellular structure and composition

The composition and cellular structure of both untreated and pre-treated pea and lupine were analysed for evaluating the influence of different types of pre-

treatment. The initial composition of pea and lupine is given in Table 12. Pea and lupine that were subjected to freeze cycles or soaking did not change in composition. Whereas about half of the lipid in pea (0.7 ± 0.1 g/100 g dry matter) and lupine (3.7 ± 0.3 g/100 g dry matter) was removed because defatting was applied to coarsely milled particles. It has been hypothesised in literature that under these conditions the content of the oil bodies is removed by defatting while the membrane lipids remain (Sánchez-Vioque, Clemente et al., 1998).

Table 12 Composition of samples ± absolute deviation (n=2).

Sample	Protein content (g/100 g dry matter)	Starch content [1] (g/100 g dry matter)	Fibre content [2] (g/100 g dry matter)	Ash content (g/100 g dry matter)	Lipid content (g/100 g dry matter)
Pea	21.9 ± 0.0	47.6 ± 1.0	23.8 ± 1.0	5.0 ± 0.0	1.7 ± 0.2
Lupine	35.1 ± 1.9		55.0 ± 1.9	2.6 ± 0.0	7.3 ± 0.2

[1] (Pelgrom, Boom et al., 2015b), [2] determined by difference

SEM imaging showed that the original cellular structure was only affected after soaking of grits (Figure 36). Originally, the cotyledon cells from pea contain large oval starch granules (± 22 µm) embedded in a matrix of small protein bodies (± 3 µm) (Pelgrom, Vissers et al., 2013) (Figure 36A). Cotyledon cells from lupine have protein bodies with a size between 5 and 25 µm (Figure 36B). Soaking of the grits led to dissolution of protein bodies (Figure 36E and F), which is undesirable for the subsequent milling and air classification. The dissolution of native pea and lupine protein may in fact be expected on the basis of previously reported high nitrogen solubilities of 85 and 100% for pea and lupine protein, respectively (Berghout, Boom et al., 2014a; Pelgrom, Boom et al., 2015b).

The firmness of pea and lupine seeds decreased after application of soaking, freezing cycles and subsequent drying of the seeds (Figure 37). Soaking and drying decreased the firmness because after drying part of the water was

replaced by air, which was observed by an increase in seed size compared to untreated seeds. Application of freezing cycles to soaked seeds was directed to the formation of (extracellular) ice crystals to inflict damage to the membranes making the seeds more fragile (Fahloul, Scanlon et al., 1996). Figure 37 shows that freezing cycles decreased the firmness of pea seeds. These pre-treatments could reduce the energy needed for milling, although application of freezing cycles probably requires more energy. Moreover, for wheat the break behaviour of soft kernels causes less starch damage (Dziki and Laskowski, 2010), which implies that more disentanglement takes place. These types of pre-treatment did not lead to any denaturation of proteins, which was confirmed by differential scanning calorimetry measurements (Figure 38).

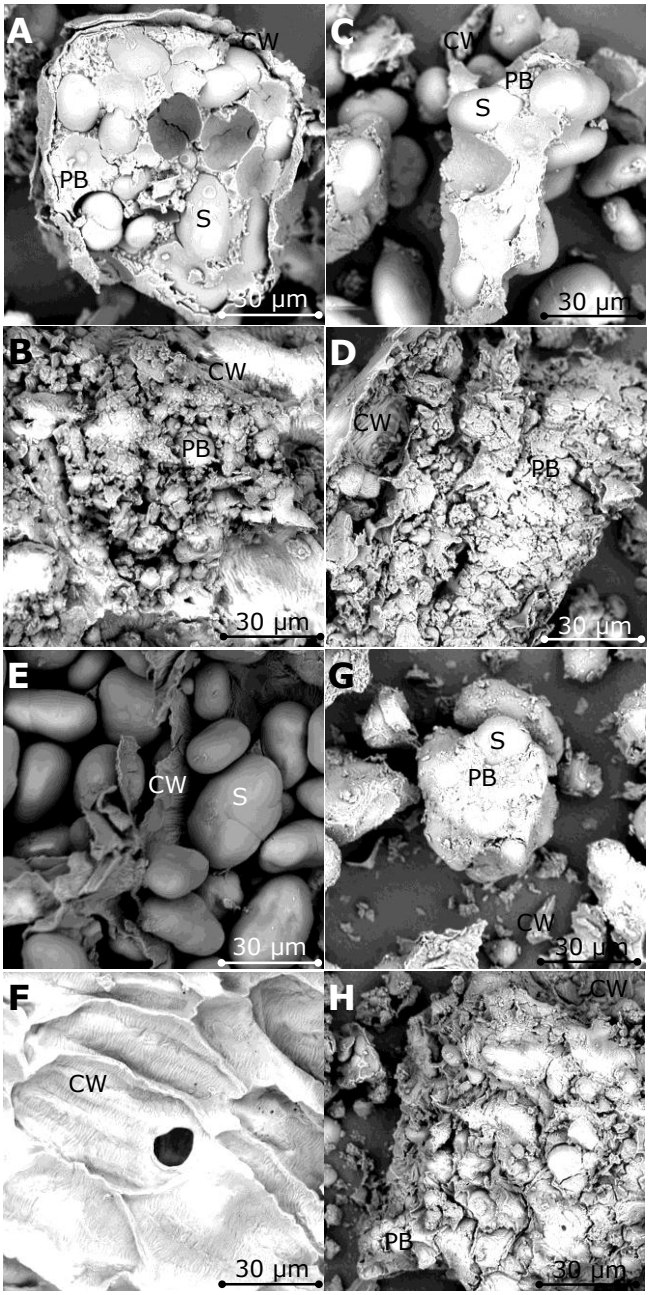


Figure 36 Cellular structure of pea cotyledon (A), lupine cotyledon (B), defatted pea cotyledon (C), defatted lupine cotyledon (D), soaked and dried pea cotyledon (E), soaked and dried lupine cotyledon (F) pea cotyledon after freezing cycles (G) and lupine cotyledon after freezing cycles (H). Protein bodies (PB), starch granules (S) and cell wall (CW) can be distinguished. In the soaked and dried samples (E and F) protein bodies were dissolved.

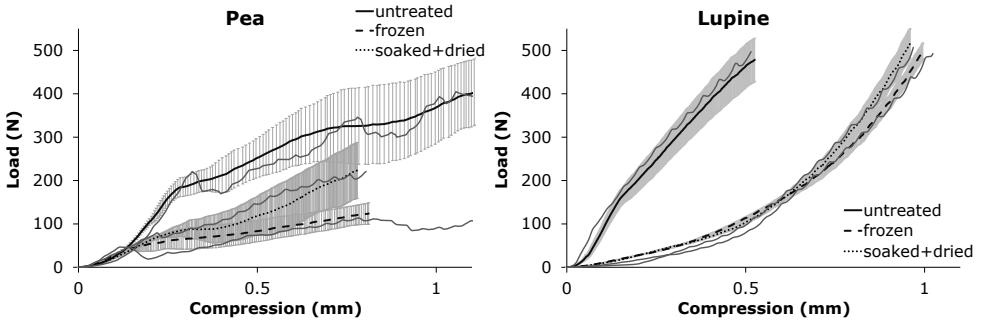


Figure 37 Break behaviour at room temperature of pea and lupine seeds that were untreated, soaked and dried or subjected to freezing cycles, which included soaking and drying. Averages of 20 measurements are provided together with the absolute deviation and a typical curve of one sample. Decreases in load indicate breakage.

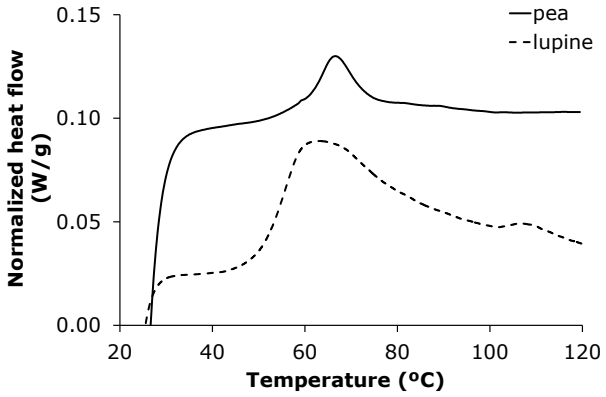


Figure 38 Typical DSC curves of pea and lupine flour.

Influence on milling

The moisture content of the milled legumes is known to affect the particles size during milling (Dijkink and Langelaan, 2002; Schorno, Manthey et al., 2009). This effect was quantified for both pea and lupine (Figure 39). A lower moisture content gives smaller particle sizes at similar milling settings, which can be explained by the increase in brittleness at low moisture content (Pelgrom, Schutyser et al., 2013). The moisture content in all other milling experiments was adjusted to the original moisture content (13 g water/100 g pea seeds and 11 g water/100 g lupine seeds).

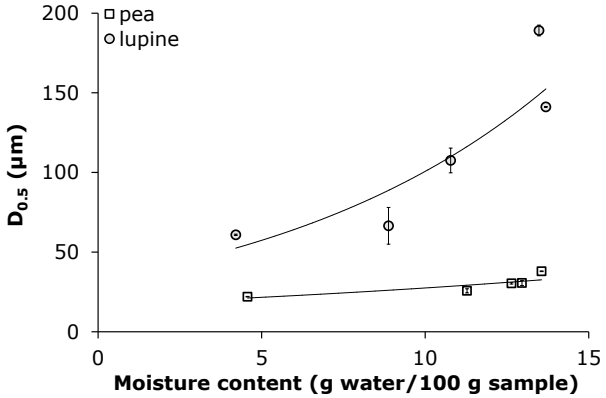


Figure 39 Particle size ($D_{0.5}$) as a function of the moisture content for pea and lupine ± absolute deviation (n=2). Lines are added to guide the eye.

During dry milling of pea, starch granules are separated from the surrounding protein matrix. The peak in the particle size distribution around 22-26 µm represents the starch granule fraction (Figure 40A). The peaks between 1 and 10 µm represent the protein bodies and parts of cell wall. Defatting, soaking or freezing cycles did not affect the particle size distribution after milling, which was expected for the starch granules as these are unaffected by the pre-treatments. The yield of the flour after milling was 85.0 ± 2.7 g flour/100 g grits for all pre-treatments.

Dry milling of lupine liberates protein bodies from the surrounding cell walls. Coarse milling was applied to avoid the production of small cell wall debris with similar size as the protein bodies. All pre-treatments led to an increase in the particle size at similar milling conditions (Figure 40B). This may be explained by the lower ($p < 0.05$) particle density after soaking (1364 ±18 kg/m^3) and after freezing cycles (1374 ± 3 kg/m^3) compared to that of the original lupine flour (1411 ± 6 kg/m^3). Particles with a low density fluidize better compared to their heavier counterparts of the same size. Therefore, more large particles are taken along by the air flow and leave the mill via the classifier wheel. The density decreased during drying because the grits and seeds did not shrink back to their original size. Absorbed water was partly replaced by air without collapsing of the structure. Besides, soaking removed the peak that was originally present at around 10 µm because protein bodies were partly extracted from the grits.

Defatted lupine flour had a similar (p < 0.05) density (1420 ± 8 kg/m^3) as original lupine flour, but due to defatting the flour was less cohesive. This decreased the time that the particles were in the milling chamber and therefore decreased the extent of milling. The yield was unaffected by the differences in particle size and was 96.6 ± 1.4 g flour/100 g seeds for all pre-treatments.

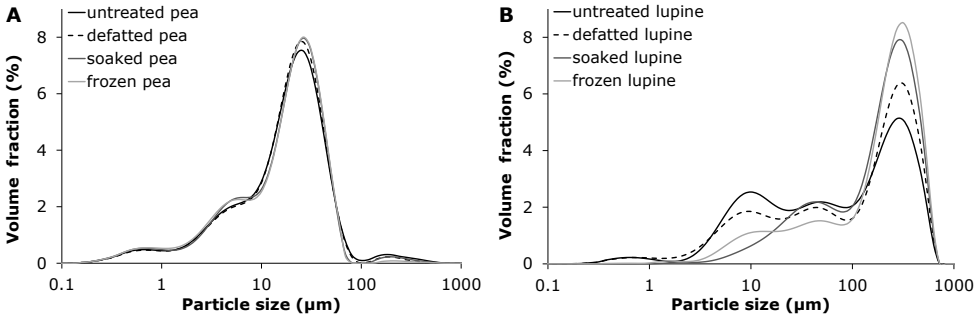


Figure 40 Particle size distributions of milled peas (A) and milled lupine (B) which were obtained after various pre-treatments. Curves represent the average of two independent measurements. The average absolute deviation was 0.07.

Influence on separation based on size and density

The milled flours that were prepared and described in the previous section were subjected to sieving or air classification to obtain a protein-enriched fraction. Samples with various moisture contents were sieved instead of air classified, because it avoided drying of the samples during air classification. Sieving with a 20 µm sieve gives similar trends as air classification (Pelgrom, Vissers et al., 2013).

Air classification can be used to obtain fine fractions with various particle sizes and protein purities. During earlier research we found that fine fractions of pea with a $D_{0.5}$ of 8.8 µm contained 51.0 ± 1.5 g protein/g dry matter, while particles of 4.8 µm contained 55.5 ± 0.7 g protein/g dry matter (Pelgrom, Vissers et al., 2013). For lupine, fine fractions with a $D_{0.5}$ of 11.4 µm contained 53.7 ± 0.9 g protein/g dry matter and particles of 6.3 µm contained 58.9 ± 0.0 g protein/g dry matter (Pelgrom, Berghout et al., 2014). However, at increased protein purity, the yield and therefore also the protein separation efficiency

(PSE) decrease, in which PSE is defined as the percentage of the total flour protein recovered in the protein fraction.

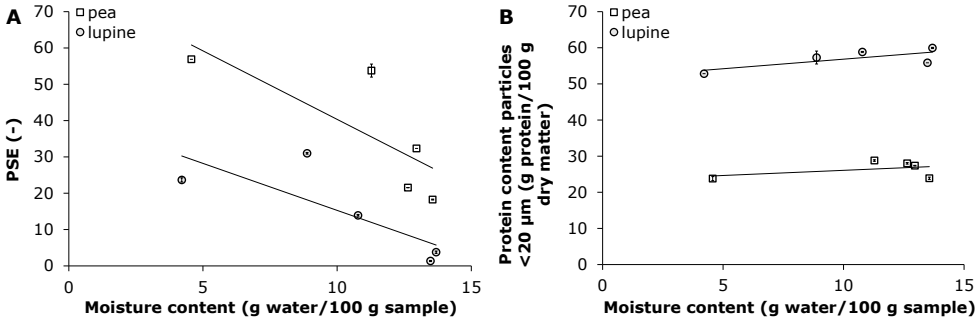


Figure 41 (A) Protein separation efficiency for particles smaller than 20 µm as a function of the moisture content for pea and lupine ± absolute deviation (n=2). (B) Protein content of particles smaller than 20 µm as a function of the moisture content for pea and lupine ± absolute deviation (n=2). Lines are added to guide the eye.

A reduction of the moisture content increased the PSE (Figure 41A) based on experiments performed with a 20 µm sieve. Lowering the moisture content increases the yield of small particles after milling due to increased brittleness of the particles. Consequently, particles smaller than 20 µm with lower moisture content had a lower protein content ($p < 0.05$) (Figure 41B). This is in line with scanning electron microscope images of Pelgrom, Schutyser et al. (2013a) that showed that milling at a lower moisture content created flat break surfaces in cells; i.e., not between protein and starch bodies, but through them, which led to less detachment of the protein bodies and starch granules or fibre. These images also showed that a higher moisture content enhanced the detachment of protein bodies and starch granules in pea (Pelgrom, Schutyser et al., 2013), which is in line with the increased ($p < 0.05$) protein content of particles smaller than 20 µm after milling at higher moisture contents (Figure 41B).

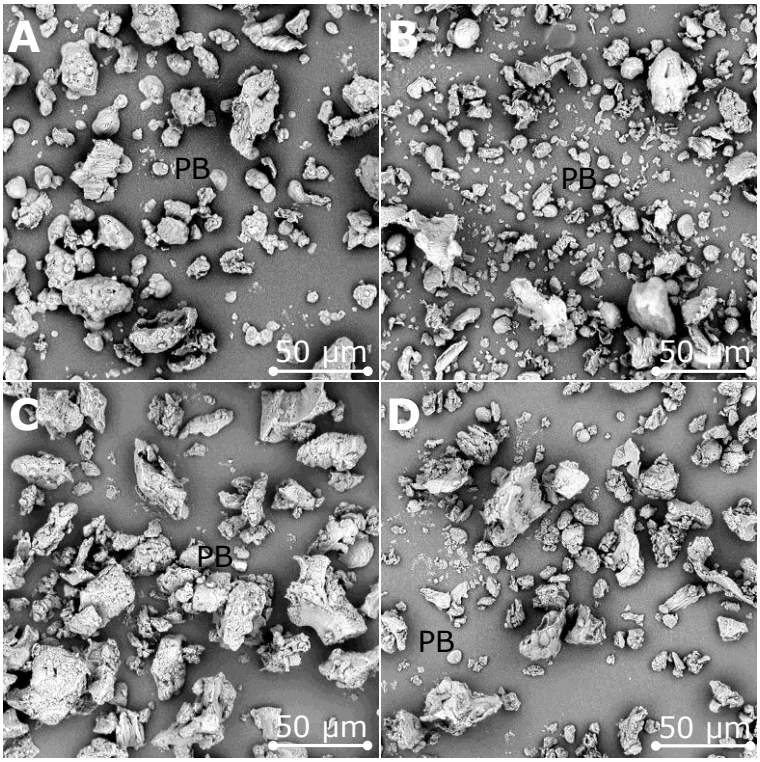


Figure 42 Cellular structure of lupine fine fraction: (A) untreated, (B) defatted (C) soaked, (D) frozen. Protein bodies are indicated by PB.

Pea and lupine grits were defatted to increase their protein content and to decrease the cohesiveness of the powder. Defatting of pea had no effect on air classification. The lipid content in pea of less than 2 g lipid/100 g dry matter was probably insignificant. However, defatting of lupine significantly ($p < 0.05$) decreased the yield and increased the protein content of the fine fraction (Table 13). The yield decreased because fewer small particles were present in defatted flour after milling. The protein content increased because smaller particles entered the fine fraction of defatted lupine compared to untreated lupine (Table 13 and Figure 42A, B). Presumably, the reduced cohesiveness caused small particles to be more dispersed. When no defatting step is applied a similar protein content can only be reached at a yield of approximately 11 g/100 g (Pelgrom, Berghout et al., 2014) compared to 21 g/100 g. The yield could potentially be increased further by altering the order of milling and defatting, i.e.

defatting lupine flour instead of lupine grits, which should result in more small particles.

Pre-soaking decreased the protein content of the fine fractions of pea and lupine and increased that of the coarse fractions (Table 13). This was expected as soaking affects the integrity of the protein bodies making subsequent air classification ineffective. Some separation could be seen because not all protein bodies were dissolved during soaking (Figure 42C). Moreover, the lower density of the particles hindered separation. During milling, the seeds were insufficiently milled to detach the components from each other or to break down protein aggregates to smaller size. This was reflected in the large particle size of the fine fraction, in the increased protein content of the coarse fraction and in the decreased protein content of the fine fraction.

The application of freezing cycles of soaked pea seeds did not affect the particle size, and had little effect on the air classification. Freezing cycles of soaked lupine seeds decreased the density of the flour and led to larger particle size of the fine fraction combined with a lower protein content of the fine fraction and a higher protein content of the coarse fraction (Figure 42D, Table 13).

In conclusion, pre-treatments increasing the protein purity were: increasing the moisture content and defatting of lupine. Protein yield was increased at lower moisture contents.

Table 13 Air classification results of pea and lupine after various pre-treatments ± absolute deviation (n=2).

Sample	$D_{0.5}$ fine fraction (µm)	Protein content fine fraction (g protein/100 g dry matter)	Protein content coarse fraction (g protein/100 g dry matter)	Yield fine fraction (g/100 g)
Pea	9.9 ± 0.5	43.9 ± 1.2	11.4 ± 1.3	32.5 ± 0.4
Pea defatted	10.6 ± 0.2	41.6 ± 0.2	11.4 ± 0.5	32.4 ± 0.6
Pea soaked	9.3 ± 0.2	34.6 ± 0.4	13.5 ± 0.5	31.3 ± 1.1
Pea frozen	9.4 ± 0.5	39.7 ± 2.0	12.7 ± 0.2	30.1 ± 1.5
Lupine	27.8 ± 5.3	45.1 ± 3.3	29.0 ± 0.8	44.4 ± 6.1
Lupine defatted	12.4 ± 1.2	56.9 ± 2.0	39.7 ± 1.4	21.3 ± 2.3
Lupine soaked	56.9 ± 3.4	43.2 ± 0.7	37.8 ± 0.3	17.9 ± 3.1
Lupine frozen	44.4 ± 9.0	43.0 ± 1.4	34.8 ± 2.5	36.5 ± 0.9

6.4.2 Post-treatment: electrostatic separation (ES)

Electrostatic separation of pea and lupine fractions

ES was used to fractionate pea and lupine flour, and their fine and coarse fractions. The hypothesis was that by using two different driving forces for separation, higher purities may be obtained than by using a single one. While the pea flour fractions did not show an increased protein content (Figure 43), lupine flour (35.1 ± 1.8 g protein/100 g dry matter) separated into a protein-enriched fraction on the grounded electrode (59.3 ± 0.2 g protein/100 g dry matter) and a protein-depleted fraction on the positive electrode (23.0 ± 0.1 g protein/100 g dry matter). Thus lupine protein presumably took a positive charge, and lupine fibres presumably took a negative charge. This is in contrast to rice, of which the proteins take a negative charge (Shih, 2003). However, aleurone cell walls and proteins from wheat bran are known to charge positively, while the fibre-rich debris of the pericarp from wheat bran charges negatively (Hemery, Holopainen et al., 2011). This indicates that the tribo-electric charging

behaviour of proteins is species dependent. Protein bodies from different origins can have different shapes, sizes and surface properties, and these differences result in different tribo-electric charging behaviour (Wang, de Wit et al., 2014).

For each air-classified fraction, electrostatic separation was used to obtain a fraction that was enriched in protein ($P<0.05$) (Figure 43). For the pea fine fraction that results from air classification, the fraction that accumulated on the grounded electrode was enriched in protein, whereas for the coarse fraction, protein was enriched on the positive electrode. This may be due to the different composition of the two fractions. The pea fine fraction contains 2 g starch/100 g dry matter and 48 g fibre/100 g dry matter. The pea coarse fraction contains 67 g starch/100 g dry matter and 23 g fibre/100 g dry matter (Pelgrom, Boom et al., 2015b). Starch granules and protein bodies were probably attracted to the grounded electrode, while fibre was more attracted to the positive electrode. These fibre particles contained also membrane proteins and components that were not detached during milling, which explains the presence of protein in the fraction on the positive electrode. Next to that, protein of the coarse fraction was enriched on the positive electrode, because this fraction was depleted in starch, which increased the percentage of the other components, such as protein that was attached to fibre particles.

For lupine, a higher purity of the fine fraction can be obtained by electrostatic separation, but even more interesting is that a fraction with similarly high protein content could be obtained from the coarse fraction (Figure 43). In agreement with this is that also from the whole flour, the same enrichment can be obtained. Therefore, electrostatic separation alone has the potential to be used as a purification process for lupine flour. Potentially, higher purity and yield can be obtained by optimization of the settings, like: air flow, particle size, voltage, and charging tube length.

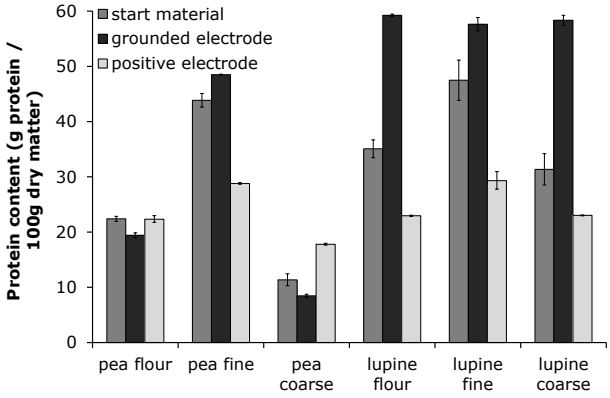


Figure 43 Electrostatic separation of pea and lupine flour and their fractions obtained by air classification ± absolute deviation (n=2).

Influence of initial protein content on electrostatic separation

The protein enrichment, defined as the ratio of the increase in protein content of the enriched fraction to that of the starting material, was further investigated by applying electrostatic separation to pea and lupine samples with various initial protein contents (Figure 44). Higher pea protein purity was reached when less starch was present as both protein and starch were attracted to the grounded electrode. The pea protein could thus not be separated from starch in this way, but it could partly be separated from fibre.

Increasing the initial protein content further decreased the separation. A reason could be that the fractions with increasing protein content were less well dispersed in the nitrogen gas flow due to Van der Waals interactions, because average pea particle size decreased from 24.3 ± 1.2 µm with a protein content of 11.4 ± 1.2 g protein/100 g dry matter to 8.0 ± 0.1 µm with a protein content of 54.4 ± 0.1 g protein/100 g dry matter. A lower dispersibility impairs the charging behaviour and thus the separation. A similar explanation holds for lupine powders with high initial protein content.

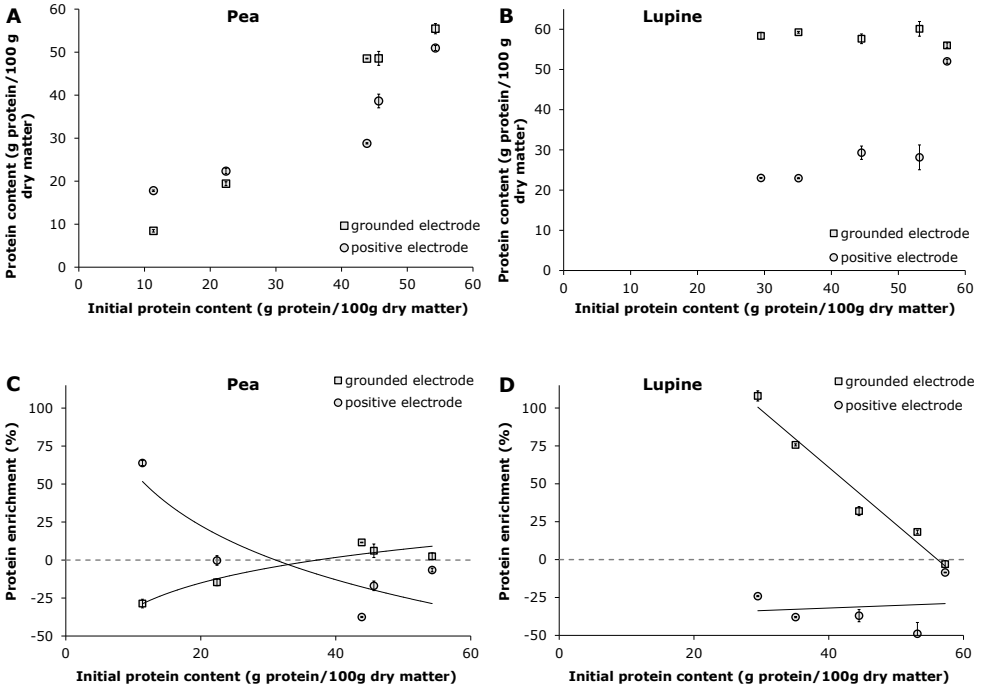


Figure 44 Protein content measured for fractions obtained from the positive and grounded electrode as a function of the initial protein content of pea (A) or lupine (B) flour and the obtained protein enrichment, ratio of protein concentration enriched, for pea (C) or lupine (D) ± absolute deviation (n=2). Lines are added to guide the eye.

6.5 Conclusions

Different types of pre- and post-treatments were combined with air classification to increase the protein purity and yield in the fine fraction obtained from pea and lupine seeds. Pre-treatments that affect the composition of pea and lupine (i.e. adjustment of the moisture content, or the removal of the hull or of the lipids by defatting) were found effective in increasing the protein content and yield with subsequent air classification. Further improvements on yield may be realised by defatting the flour after milling or by optimising air classification settings for density changes as a result of pre-treatment. Electrostatic separation was evaluated as a post-treatment and increased the protein content of all air-classified fractions. Since the two types of seeds that were investigated represent two different major classes of legumes (starch based and oil based), electrostatic separation can probably be used to prepare protein concentrates from a wide range of legumes.

6.6 Acknowledgements

The authors wish to thank The PEAS Foundation, the Dutch Technology Foundation STW and ISPT for project coordination support through the Intensified Protein Structuring project (PI-00-03) and the DRYFRAC project (grant number 11399). We acknowledge Ying Zhao for her support in performing experimental work and Atze Jan van der Goot for his project support.

7 Dry fractionation of starch-rich legumes

This chapter has been submitted as: Pelgrom, P.J.M., Boom, R.M., Schutyser, M.A.I. (2015) Dry fractionation of starch-rich legumes.

7.1 Abstract

A facile method to establish milling settings that optimally separate starch granules from protein bodies and cell wall fibres was developed for starch-rich legumes. Optimal separation was obtained for pea, bean, lentil and chickpea when the particle size distribution curve of flour and isolated starch granules overlap maximally. This outcome was based on scanning electron microscopy, protein content of the fine fraction and particle size distribution curves. Milling settings differed between legumes due to variances in seed hardness and starch granule size. The protein content of the fine fraction was legume specific as well and could be explained by differences in particle density, seed hardness, starch granule size, fat content and flour dispersibility.

7.2 Introduction

Interest in legume proteins has increased in recent years due to their nutritional and functional properties and for sustainability reasons (Rahman, 2006; Boye, Zare et al., 2010). Legume protein concentrates are low in fat and are an excellent sources of protein, dietary fibre and a variety of micronutrients and phytochemicals (Boye, Zare et al., 2010; Pelgrom, Boom et al., 2015a). These protein have been used in food products for their solubility or their gelation and dough formation capacity (Rahman, 2006). Moreover, consumption of legume proteins instead of animal protein would lead to a more efficient and more sustainable food supply (Passe, Fouache et al., 2008; Aiking, 2011).

Wet fractionation is conventionally used to purify plant proteins, however this process uses large amounts of water and energy. Furthermore, the native functionality of the proteins is lost due to pH changes and elevated temperatures during dehydration (Schutyser and Van der Goot, 2011). An alternative process to enrich plant proteins is dry fractionation, which is carried out by milling and air classification. Milling detaches the starch granules from smaller protein-rich particles. During subsequent air classification, the starch granules and protein fragments are separated on their difference in density and size.

Legumes that have been subjected to dry fractionation are peas, mung beans, lentils, common beans, faba beans, navy beans, lima beans, cowpeas. Their protein enrichment is facilitated by the size difference between protein-rich particles (approx. 5 µm) (Pernollet, 1978) and starch granules (approx. 15-40 µm) (Watanabe, Tang et al., 1996). The protein content of the fine fraction varies between 49 g/100 g dry matter for lima beans and 70 g/100 g dry matter for faba beans (Sosulski and Youngs, 1979; Tyler, Youngs et al., 1981; Elkowicz and Sosulski, 1982). Protein enrichment is enhanced by larger starch granule size (Tyler, 1984; Cloutt, Walker et al., 1987).

To obtain maximum protein enrichment, the degree of milling should be such that starch granules and protein are detached. However, very fine milling is not optimal for air classification; the non-protein components, like starch and fibre,

should remain larger than the protein bodies (Pelgrom, Vissers et al., 2013). A better understanding of the particle size distribution in relation to detachment would enable us to predict protein enrichment after air classification and to extent knowledge on milling of pea to a wider range of starch-rich legumes.

In this study milling is optimised towards maximum detachment of starch granules, in contrast to previous studies in which all legumes were milled at a single setting (Sosulski and Youngs, 1979; Tyler, 1984; Cloutt, Walker et al., 1987). We explore the hypothesis that optimal detachment is reached when the particle size distribution curve of flour overlaps maximally with the particle size distribution curve of the starch granules. The added value of this hypothesis is that optimal milling conditions and thus detachment settings for a specific starch-rich legume can be obtained easily, thereby facilitating protein enrichment of these crops by subsequent air classification.

Although our hypothesis is based only on maximum overlap between starch granule and flour size, other factors have been reported to influence optimum detachment as well. Tyler (1984) described that the protein separation efficiency (PSE), defined as the proportion of the total flour protein shifted into the fine fraction during air classification, was negatively correlated to seed hardness, crude fibre content and water insoluble cell wall content of the seed. In contrary to Tyler (1984), Wu found that the separation efficiency improved for softer wheat seeds (Wu, 1992). The relation between crude fibre content and protein separation efficiency is debatable as well, as other measures for the amount of cell wall material, like neutral detergent fibre content and cell wall thickness were not related to protein separation efficiency. Next to that, Tyler (1984) reported that levels of protein, starch and ash had little or no effect on the impact milling characteristics of the legumes, while the protein content in pea was positively correlated with the protein content in air-classified fractions (Reichtert, 1982). Another factor that influences the separation efficiency of legumes is the amount of oil present. Chickpea, lupine and soy have been reported to be unsuitable for dry fractionation (Sosulski and Youngs, 1979; Elkowicz and Sosulski, 1982). However, we were recently able to enrich lupine

flour in protein by adapting the milling settings to the seed morphology (Pelgrom, Berghout et al., 2014).

Concluding, although maximum overlap is proposed as route for optimal detachment, it does not cover all aspects that determine optimal milling conditions of legumes and subsequent effective separation. Therefore, four starch-rich legumes, i.e. pea, bean, chickpea and lentil, were extensively characterised and their properties were correlated to results of the dry separation. First, the composition of the legumes, their morphology and starch granule size were characterised. Then, to validate our approach, legumes were milled and air classified. The fractions were tested for protein content, particle size distribution, particle density and dispersibility.

7.3 Materials and methods

7.3.1 Materials

Pre-dried chickpea, *Cicer arietinum*, Lentil, Lens *culinaris*, Pea, *Pisum sativum*, and Bean, *P. vulgaris*, were purchased from Alimex (Sint Kruis, The Netherlands). All materials still contained their hulls and were unheated. Experiments were done at least in duplicate.

7.3.2 Milling, sieving and air classification

Legume were pre-milled to grits ($D_{0.5}$ of 140 - 220 µm) with a Condux-Werk LV 15M (Condux-Werk, Wolfgang bei Hanau, Germany). The grits were milled with a ZPS50 impact mill (Hosokawa-Alpine, Augsburg, Germany). This mill contains an internal rotating classifier wheel that allows the passage of fine particles while coarse particles are returned and further milled. The classifier wheel speed determined, together with the air flow, the size of the milled flour. The classifier wheel was varied between 2200 and 8000 rpm and the air flow was varied between 40 and 52 m^3/h. Other parameters were: a feed rate of 2 rpm (circa 0.5 kg/h), an impact mill speed of 8000 rpm and a batch size of at least 600 g.

Pea flour was separated by air jet sieving (Alpine200 LS-N, Hosokawa-Alpine, Augsburg, Germany) during 2.5 minutes at 4000 Pa on a 20 µm sieve. Each experiment started with 9.9 g of flour, which was mixed with 0.1 g fumed silica

(Aerosil®200, Azelis Netherlands B.V., Oosterhout, The Netherlands) to improve the flowability.

All flours were air classified in an ATP50 classifier (Hosokawa-Alpine, Augsburg, Germany). The air flow was fixed at 52 m^3/h. The classifier wheel speed was set at 5000 or 10000 rpm. The feed rate was set at 15 rpm (circa 1.0 kg/h). Per batch at least 160 g flour was air classified.

7.3.3 Compositional analyses

The dry matter content was determined by drying 1 gram of sample overnight in an oven at 105ºC.

The protein content was analysed using Dumas analysis (Nitrogen analyzer, FlashEA 1112 series, Thermo Scientific, Interscience, Breda, The Netherlands). A nitrogen conversion factor of 6.25 was used.

The fat content was measured in a fully automated Büchi extraction system B-811 LSV (Büchi Labortechnik AG, Flawil, Switzerland). Fat extraction was performed with petroleum ether (boiling range 40-60ºC) in Standard Soxhlet mode for 3 hours with a sample-to-solvent ratio of 1:6.

7.3.4 Particle analyses

The particle size distribution of the samples was determined by laser diffraction using a Mastersizer 2000 equipped with a Scirocco 2000 dry dispersion unit (Malvern Instruments, Worcestershire, UK). Pressure of 400 kPa was used and the volume-weighted particle size distribution was calculated using the Fraunhofer theory.

The dispersibility of the flours was measured according to (Pelgrom, Berghout et al., 2014). The ratio between the particle size at a pressure of 50 and 400 kPa was determined using a Mastersizer 2000 equipped with a Scirocco 2000 dry dispersion unit (Malvern Instruments, Worcestershire, UK).

The scanning microscope images were obtained with a Phenom G2 Pure (Phenom-World BV,Eindhoven, the Netherlands) according to (Pelgrom, Berghout et al., 2014).

The particle density of all legume grits was measured using a pycnometer (Ultrapyc 1200e, Quantachrome Instruments, Boynton Beach, USA) operating with nitrogen.

The seed hardness of all legume seeds was determined according to (Pelgrom, Boom et al., 2015c). Twenty cotyledons per legume were, flat-side down, compressed by a 15 mm cylindrical probe attached to a Texture Analyser (Instron-5564Series-Table-Model- Systems-Twin-column-design, Canton, USA) equipped with a 2000 N load cell at a crosshead speed of 20 mm/min.

7.3.5 Starch isolation

Starch granules were isolated by steeping 50 g of the seeds in excess tap water overnight at 4ºC. The seeds were milled for 2 minutes combined with the steep water in a domestic blender (Philips HR7776/90, Philips, Eindhoven, The Netherlands). The slurry was washed with 500 ml tap water on a 125 µm and a 90 µm sieve. The sieving water was collected and allowed to settle for 1 h at 4ºC. The white bottom layer was collected and re-dispersed in 100 ml tap water. The suspension was centrifuged for 20 min at 3000 g. The supernatant and a non-white layer were removed. The white bottom layer was dispersed again in 100 ml tap water and the centrifugation procedure was repeated 2 times. Part of the pellet was dried in a vacuum oven at 40ºC overnight. The other part was suspended in tap water and stored for particle size distribution analysis.

The particle size distribution of starch granule suspensions was analysed by laser diffraction using a Mastersizer particle size analyser (Malvern Instruments Ltd. 2000, Worcestershire, UK) according to (Pelgrom, Boom et al., 2015b).

The total starch content of isolated starch from the four legumes was determined using a Total Starch Amyloglucosidase/a-Amylase Assay Kit

(Megazyme International Ireland Ltd, Bray, Ireland). The starch isolate contained 92 to 96 g starch/100 g dry matter.

7.3.6 Statistical analysis

Student's-*t* tests were performed to evaluate the differences between fractions. Differences were considered to be significantly different when the *p*-value was smaller than 0.05.

7.4 Results and discussion

The particle size distribution (PSD) curves of pea flours and wet isolated starch were first analysed to explore the hypothesis that optimal detachment is reached when the overlap between the particle size distribution curve of the starch granules and that of the pea flour is maximal. The overlap for pea is maximal at a classifier wheel speed of 4000 rpm during milling (Table 14).

The hypothesis that this is an indicator for optimal detachment of the starch granules from the protein matrix and the cell wall fibre was confirmed by assessing the composition of the particles smaller than 20 µm by sieving (Table 14). Milling seeds at 2500 rpm created insufficient detachment, while flour milled at 8000 rpm had similar protein content of particles smaller and larger than 20 µm due to the small size of the flour particles, and thus showed no potential for separation anymore.

The optimal milling settings are in agreement with previous, intuitively optimised, settings for milling and air classification of pea (Pelgrom, Vissers et al., 2013).

The hypothesis is further evaluated for bean, chickpea and lentil in the next section.

Table 14 The effect of classifier wheel speed during milling on flour size and protein enrichment of pea ± absolute deviation (n=2) (Pelgrom, Vissers et al., 2013).

Classifier wheel speed (rpm)	$D_{0.5}$ (µm)	Overlap with starch granule curve (%)	Protein enrichment particles <20µm (%)
2500	19.3 ± 0.7	58.1 ± 1.0	35.6 ± 9.1
4000	17.9 ± 0.7	63.2 ± 2.2	51.3 ± 3.7
8000	8.0 ± 0.1	21.5 ± 0.9	-2.9 ± 1.8

7.4.1 General comparison of the four legumes

Figure 45 shows that the cotyledon architectures of the four legumes are similar. Starch granules (S) are embedded in a matrix of protein bodies (P) and are surrounded by a fibre-rich cell wall (CW). The starch granules of pea, bean and lentil are around 25 µm, while chickpea starch granules are smaller with a size of 18 µm (Table 15), which is in agreement with literature (Hoover and Ratnayake, 2002). Other differences between the legumes were the higher overall protein content of bean (p<0.05) and the higher fat content of chickpea (p<0.05) (Table 15).

The $D_{0.5}$ of bean and lentil after milling at fixed settings were significantly (p<0.05) lower than that of pea (Table 15). This can be explained by the lower seed hardness of bean and lentil (Table 15). The $D_{0.5}$ of chickpea was similar to that of pea; however the starch granules of chickpea are smaller. Thus, to obtain maximum overlap between the particle size distribution of starch granules and milled flour more intensive milling is expected to be needed.

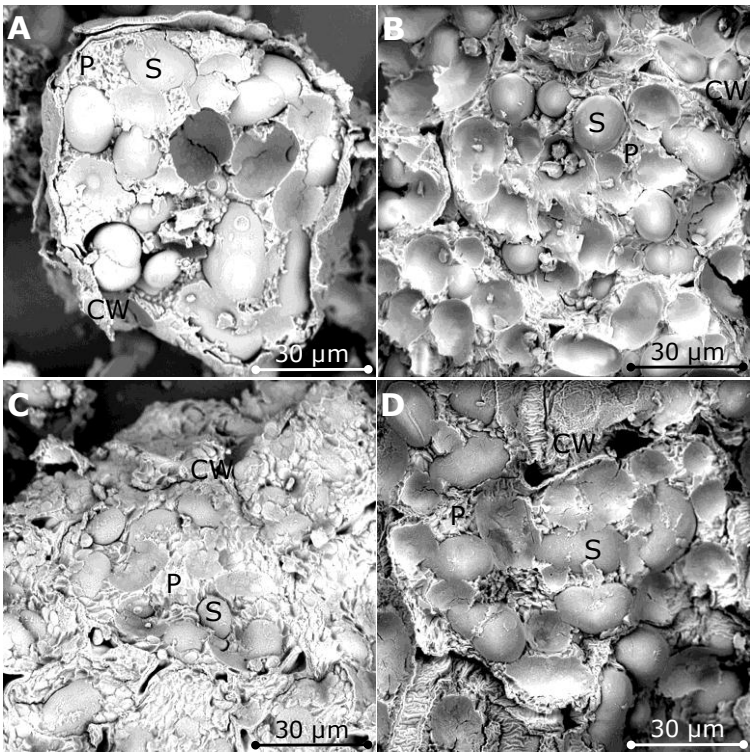


Figure 45 Morphology of pea (A), bean (B), chickpea (C) and lentil (D). Starch granules (S), protein bodies (P), and cell wall (CW) can be distinguished.

Table 15 Properties of the four legumes that may be of influence to the disentanglement and subsequent protein enrichment of the legumes. The particle size was determined after milling at 2900 rpm ± absolute deviation (n=2).

Legume	Starch granule size (µm)	Protein content (g/100g dry matter)	Fat content (g/100g dry matter)	Moisture content (g/100g sample)
Pea	25.8 ± 0.5	23.7 ± 0.8	1.9 ± 0.3	12.6 ± 0.4
Bean	25.0 ± 0.6	29.8 ± 1.4	2.0 ± 0.2	12.7 ± 0.1
Chickpea	18.9 ± 0.1	21.6 ± 0.9	6.6 ± 0.2	11.7 ± 0.1
Lentil	25.1 ± 0.8	24.9 ± 0.3	2.1 ± 0.0	11.9 ± 0.8

Table 15 continued

Legume	$D_{0.5}$ (µm)	Seed hardness (N)	Particle density (kg/m^3)
Pea	17.1 ± 0.8	210 ± 23	1441 ± 4.5
Bean	14.9 ± 0.2	126 ± 13	1427 ± 2.7
Chickpea	16.1 ± 0.4	197 ± 28	1408 ± 2.8
Lentil	12.4 ± 0.9	31 ± 18	1437 ± 0.6

7.4.2 Optimal enrichment following the maximum overlap hypothesis

The milling settings and specifically the settings of the classifier wheel were adjusted to obtain optimal detachment for all four legumes, which corresponded to the maximum overlap of the particle size distribution of the milled flour and that of the starch granules (Figure 46). The air flow was kept constant at a value of 40 m^3/h, since at this air flow rate, the width of the particle size distribution of the flour was smallest.

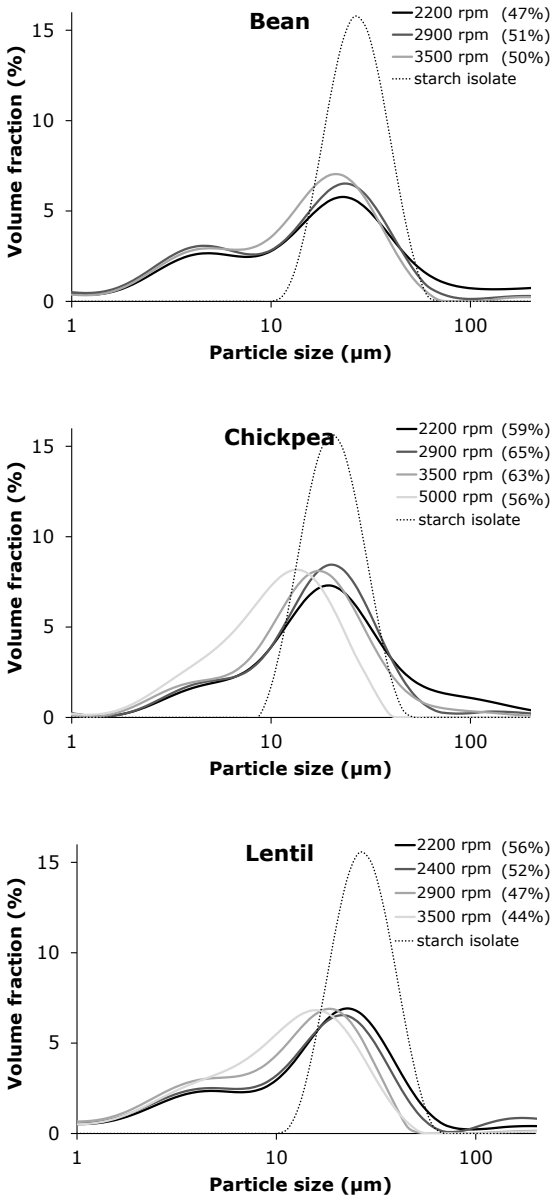


Figure 46 Particle size distribution of bean, chickpea and lentil milled at various classifier wheel speeds and the particle size distribution of their starch granules. Next to the classifier wheel speed during milling the percentage of overlap with the particle size distribution of the starch granules is given.

Figure 46 shows that the average particle size and the width of the size distribution decreased at higher classifier speeds. The detachment at milling

settings with maximum overlap in particle size distribution curves was analysed by scanning electron microscopy (SEM; Figure 47). The starch granules and smaller (probably protein matrix) fragments were separate particles and were not linked anymore. Subsequently, the flours with maximum overlap were subjected to air classification.

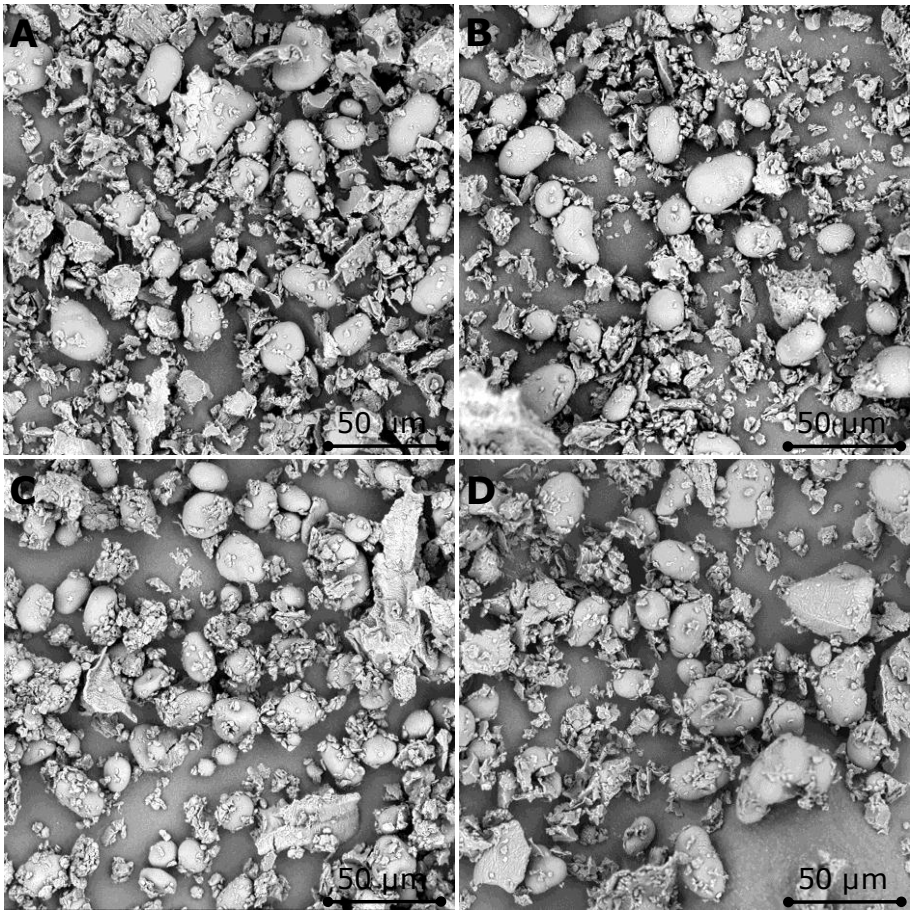


Figure 47 Electron scanning microscope images of pea (A), bean (B), chickpea (C) and lentil (D) flour milled at settings that gave maximal overlap with the particle size distribution curve of their isolate starch granules.

Air classification was performed at settings that provided a fine fraction with a smaller average particle size ($D_{0.5}$ 5.9 µm, $D_{0.9}$ 17.0 µm) than the average size of the starch granules ($D_{0.5}$ 23.7 µm, $D_{0.1}$ 15.4 µm) (Table 16). Thus, we

assumed that the fine fraction contained little starch. This assumption can only be made when flour is not milled smaller than the size of the starch granules. The low particle size of the fine fraction led to a protein content which was in agreement with the maximum protein contents reported in literature (Sosulski and Youngs, 1979; Tyler, Youngs et al., 1981; Elkowicz and Sosulski, 1982). Moreover, the particle size distribution curve of the fine fraction confirmed that smaller, protein-rich fragments were effectively separated (Figure 48). The particle size distribution curves show as well that insufficiently milled flour provided a smaller peak at 5 µm and gave a lower yield after air classification, but similar protein content (for example: 52.9 g/100 g dry bean fine fraction). Flour milled too fine contained more non-protein particles that were milled fine, which resulted in a slightly lower protein content of the fine fraction (for example: 57.4 ± 0.9 g/100 g dry lentil fine fraction compared to 58.5 ± 0.2 g/100 g dry lentil fine fraction).

Therefore, it can be concluded that the maximum overlap hypothesis provides optimal dry fractionation. However, the differences between the fine fraction protein contents of the different types of legumes cannot be explained by the hypothesis as these are also function of the material properties.

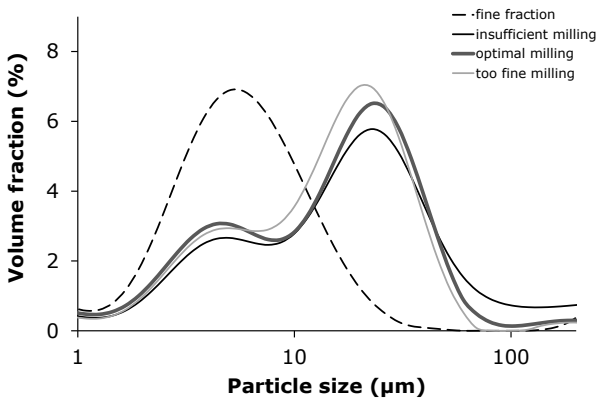


Figure 48 Particle size distribution of flours and the fine fraction of bean.

Table 16 Protein content and particle size parameters of pea, bean, chickpea and lentil air classified at 10000 rpm with an air flow of 52 m^3/h. ± absolute deviation (n=2).

Legume	Protein content fine fraction (g/100 g dry matter)	Protein content fine fraction (g/100 g dry matter) literature *	D0.5 (µm) fine	Overlap PSD fine and flour (%)
Pea	55.6 ± 0.5	58.9 ± 3.0	5.4 ± 0.0	49.0 ± 1.1
Bean	52.8 ± 0.3	55.2 ± 2.2	5.1 ± 0.1	57.8 ± 0.0
Chickpea	45.3 ± 0.7	28.9	7.8 ± 0.4	61.1 ± 2.2
Lentil	58.5 ± 0.2	57.3 ± 5.3	5.3 ± 0.2	51.0 ± 1.1

** (Sosulski and Youngs, 1979; Tyler, Youngs et al., 1981; Elkowicz and Sosulski, 1982)*

7.4.3 Legume properties that co-determine the dry enrichment of proteins

In this section the material properties of the four legumes are related to the milling and air classification results. Detachment of starch granules, protein bodies and cell wall during milling was evaluated using particle size distribution curves and seed hardness. Parameters that are related to the sharpness of air classification are: overlap in particle size distribution between the fine and the coarse fraction, particle density, starch granule and protein body size, and dispersibility.

Properties related to detachment

The protein purity of the fine fraction decreased with increasing overlap between the particle size distribution curve of the fine fraction and the flour (Table 16). A high overlap indicates that fibre and possibly starch granules are milled too fine and may enter the fine fraction. The legumes contained around 25 g protein/100 g dry matter, which meant that if the fine fraction would consist solely of protein, a smaller overlap than 50 % would be expected. The overlap was larger for bean and chickpea, because bean had to be milled smaller, and because the particle size of the fine fraction of chickpea was larger.

The higher protein content in the fine fraction may be related to the lower seed hardness of lentils (Table 15). The seed hardness is related to the adhesion between the protein matrix and starch granules (Dziki and Laskowski, 2010). Next to that, seed hardness is associated with the type and amount of insoluble fibres (Nicholls, Appleqvist et al., 1995). Seed hardness could thus be an indicator of the composition of particles of different sizes after milling. Tyler (1981) speculated that harder seeds contain a higher level of agglomeration of starch granules and protein bodies in legumes, although he found a higher protein separation efficiency for harder legumes. In contrast, Wu & Stringfellow (1992) found higher protein separation efficiency for softer wheat varieties.

Properties related to sharpness of air classification

The overlap in particle size distribution between the fine and the coarse fraction indicates the separation sharpness. For pea, bean and lentil the overlap was 24.5 ± 0.8 % but for chickpea the overlap was 53.8 ± 1.9 %. Chickpea thus gave less separation and a 11% lower yield compared to pea, bean and lentil, which may be related to the higher fat content and low density of chickpea (Table 15) impairing air classification (Sosulski and Youngs, 1979). Moreover, the chickpea starch granules were smaller providing a smaller difference in size with protein bodies (± 5 µm), which decreased separation sharpness and caused a lower protein content in the fine fraction.

Next to that, the size of the protein bodies could be of influence. The particle size distribution of bean and lentil showed a more distinctive peak at 4-5 µm compared to pea and chickpea at all milling speeds (Figure 46 and Figure 49A). This peak could be related to the size of the protein bodies, which is larger for bean (2-22 µm) than for pea (1-3 µm) (Pernollet, 1978). However, we here did not observe a clear relation between protein body size and protein enrichment, probably because other differences between legumes obscured any effect of protein body size.

Air classification separates on the basis of particle size and particle density. The particle density of chickpea was significantly ($p < 0.05$) lower than that of pea,

bean and lentil (Table 15). Consequently, larger chickpea flour particles entered the fine fraction, thereby lowering the protein content.

Finally, the dispersibility of chickpea was lower compared to pea, bean and lentil flour of similar size (Figure 49B). The elevated fat content (6.6 g/100 g dry matter compared to 2 g/100 g dry matter) may have caused the decrease in dispersibility. Moreover, the dispersibility decreased as a function of the average particle size. Lentil flour, which was most dispersible, yielded the highest protein content in the fine fraction. Bean flour, which was less dispersible than lentil and pea, yielded lower protein content in the fine fraction (Table 16).

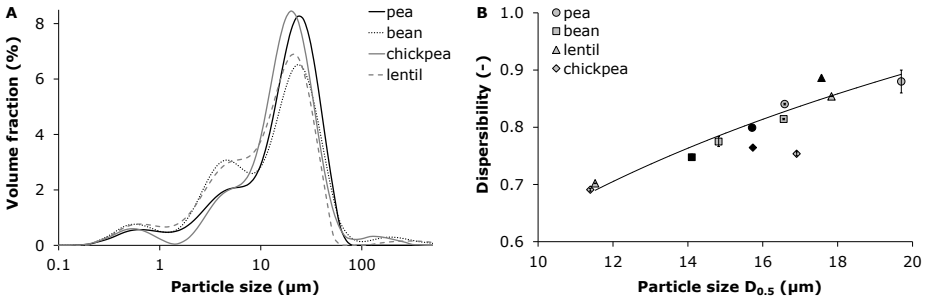


Figure 49 (A) Particle size distribution of pea, bean, chickpea and lentil milled at 2900 rpm. (B) Dispersibility as a function of particle size of pea, bean, chickpea and lentil flour. Dark data points represent the flours that were air classified. Line is added to guide the eye ± absolute deviation (n=2).

7.4.4 Routes to improve dry enrichment of proteins

The parameters that influence milling and air classification of various legumes are the basis of possible routes to improve dry fractionation. Despite differences in starch granule size, protein body size, initial protein content and seed hardness, the relation between particle size and protein content was similar for all legumes (Figure 50). This finding was in contradiction with expectations that differences in protein content of the fine fraction would be related to differences in break behaviour of the seeds; i.e. that fibres of lentil remained larger than fibres of bean. For all legumes small particles were rich in protein. Therefore, air classification to smaller sizes in the fine fraction or sharper separation will increase the protein content, however at the expense of the yield. Besides,

milling of the fine fraction to obtain finer particles could increase the protein content, although decrease in dispersibility could impair separation. To facilitate air classification of small particles redesign of the air classifier to decrease the amount of material build-up would be a solution.

Other routes to improve dry fractionation may be found in plant breeding or pre-treatment techniques. Plant breeding could focus on selection of varieties with larger starch granules, or tougher fibres or varieties with a lower seed hardness. The latter could contribute to easier detachment between starch granules and protein bodies, which could increase the particle size of the flour thereby increasing dispersibility and enhancing protein-enrichment. Pre-treatments could also be used to accomplish these changes in legume morphology.

Figure 50 furthermore shows that extrapolation of the protein content of the fine fraction leads to a maximum of 74 g protein/100 g dry matter, which is in agreement with the protein content of protein bodies that is between 70 – 88 g protein/100 g dry matter (Weber and Neumann, 1980; Plant and Moore, 1983).

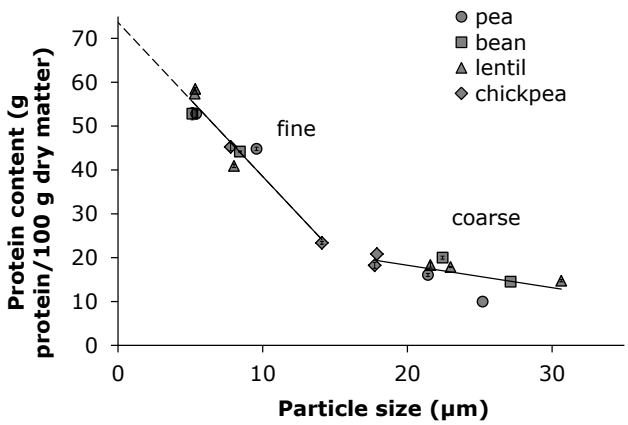


Figure 50 Protein content as a function of particle size for air classified legumes ± absolute deviation (n=2). Lines are added to guide the eye.

7.5 Conclusions

Optimal detachment was reached between starch granules and protein- and fibre-rich particles from pea, bean, chickpea and lentil by selecting those milling settings that yield the largest overlap between the particle size distribution curve of starch granules and of flour. This method is thus a facile approach to find milling settings that provide optimal detachment for starch-rich legumes. However, seed properties like seed hardness, particle density, starch granule size, fat content and flour dispersibility influence the protein content of the fine fraction as well.

It is to be expected that the maximal overlap hypothesis can be applied on a wide range of starch-rich legumes. Application on grains should also yield detachment, but subsequent air classification will probably not give large enrichments due to the small size difference between starch granules and protein bodies.

Further research could focus on pre-treatments and selection of legume varieties that possess characteristics needed for dry separation.

7.6 Acknowledgements

The authors wish to thank The PEAS Foundation and ISPT for project coordination support through the Intensified Protein Structuring project (PI-00-03). We acknowledge Atze Jan van der Goot for his project support.

8 General discussion

This chapter is part of a review publication and is submitted as: Schutyser, M.A.I., Pelgrom, P.J.M, van der Goot, A.J., Boom, R.M. (2015) Dry fractionation for sustainable production of functional legume protein concentrates.

8.1 Introduction

Production of more sustainable protein-rich foods is desirable from long-term economic and sustainability perspectives. The growing world population and the increasing consumption of animal protein impose a serious challenge for our future food supply, and a transition from animal to plant protein sources is part of the solution to cope with limited availability of resources (i.e. land and water). Legumes have gained increased interest in recent years as a source of plant proteins due to their nutritional and functional properties (Rahman, 2006; Boye, Zare et al., 2010). However, alternative routes for plant protein extraction should be investigated; routes that have less waste, consume less water, chemicals and energy. Therefore, this thesis described a dry fractionation method for legumes to obtain functional protein-enriched fractions. The aim was to increase our understanding of both the material properties of legume seeds, and of the process conditions relevant to combined milling and air classification of legumes, and from this explore more sustainable routes for production of functional ingredient protein fractions. A discussion of the main results answering this aim is given in the next paragraph.

8.2 Discussion of main findings

Dry fractionation of legumes involved fine milling and subsequent air classification. Milling aims at optimal disentanglement of protein bodies and cellular components. Insufficient milling of pea ($D_{0.5}$ > 40 µm) led to incomplete disentanglement, whereas too extensive milling ($D_{0.5}$ < 10 µm) resulted in increased starch damage and poor dispersibility of the flour (**chapter 2**). Milling settings for optimal disentanglement were further specified in the maximum overlap hypothesis, which states that disentanglement is reached when the particle size distribution curve of flour overlaps maximally with that of isolated starch granules (**chapter 7**). For four starch-rich legumes, i.e. pea, bean, chickpea and lentil, this hypothesis was confirmed. Based on starch granule size and seed hardness, the milling settings were adjusted to reach optimal disentanglement. Subsequent air classification provided a protein purity that corresponded to the maximum values reported in literature. The protein content

of the fine fraction varied per legume due to their inherent differences in particle density, starch granule size, dispersibility, and fat content.

Milling of lupine, an oil-rich legume that contains hardly any starch, should not be optimised for disentanglement of protein bodies and starch granules, but for disentanglement of protein bodies while minimizing the size reduction of the fibre particles (**chapter 5**). This strategy yielded a protein concentrate with 54-59 g protein/100 g dry matter with a yield of 6-13 g/100 g. The dispersibility of lupine flour was increased by adding fumed silica or potato starch, which both decrease the cohesive forces between lupine flour particles. Addition of fumed silica doubled the protein separation efficiency. However, fumed silica increased the dispersibility of both protein bodies and fibre particles and thus led to a decrease in protein purity.

Combining pre- and post-treatments with air classification increased the protein purity of both pea and lupine. The effect of moisture content and temperature on the disentanglement of pea protein bodies and starch granules was investigated in depth with the help of glass transition curves (**chapter 3**). Three regions existed in the relevant area in the state diagram: glassy protein and starch, rubbery protein and glassy starch and, rubbery protein and starch. Glassy pea broke at smaller forces and broke along a smooth fracture plane. In the fracture plane of pea containing rubbery protein, more disentangled starch granules could be observed. These differences were confirmed by milling experiments (**chapter 6**). Increased moisture content during milling increased the protein content of particles smaller than 20 µm due to better disentanglement. A decrease in moisture content increased the yield of particles smaller than 20 µm due to the lower energy needed for breakage. Pre-treatments that affected the composition of pea and lupine were found most effective in increasing the protein content or yield. Defatting of lupine increased the protein content as well. However, pre-treatments that involved wetting of the flour were less successful.

Electrostatic separation was applied as a post-treatment to combine a separation mechanism based on electrostatic charging behaviour of the components with a

separation mechanism based on differences in size and density (**chapter 6**). Electrostatic separation charged protein and starch positively, whereas fibre was charged more negatively. Therefore, all air-classified fractions from pea and lupine could be enriched in protein. Moreover, it was found that electrostatic separation can be used as a novel method for lupine protein enrichment from flour directly.

The properties of air-classified fractions were compared to commercial wet-extracted protein concentrates. In contrast to the wet-extracted concentrates, air-classified pea fractions contain highly soluble proteins. A liquid protein dispersion with 26 g dissolved protein/100 g sample could be prepared (**chapter 2**). This makes air-classified pea fractions an interesting ingredient for high protein drinks. The high solubility of protein also causes phase separation between protein, starch granules and fibre. Based on this property an aqueous phase separation method was proposed (**chapter 4**). A protein purity of 77 g protein/100 g dry matter was obtained at similar protein yield (63 g protein/100 g initial protein) compared to wet fractionation requiring less water and energy. The air-classified lupine fractions also contained highly soluble proteins suggesting opportunities for the development of high protein beverages as well (**chapter 5**). The high solubility enabled the formation of more stable foams compared to heat-treated lupine protein concentrates. Moreover, *in vitro* digestion experiments showed a larger amount of small peptides compared to heat-treated lupine protein concentrates.

Besides, the application of air-classified pea fractions to prepare solid protein products, like gels or meat replacers, was analysed. After heat treatment, a high water holding capacity (WHC) of 4.8 g water /g flour was obtained for the starch-rich fraction (**chapter 2**). Heat treatment induced gel formation, in which the gel strength was mainly determined by the starch content (**chapter 4**). Increasing heating rate or reducing cooling rate increased the gel strength by forming a more homogeneous gel and a stronger protein network. Transglutaminase was used to cross-link protein-rich gels. The presence of starch and fibre that absorbed water increased the gel strength compared to commercial protein isolates. In conclusion, the functional behaviour of dry-

enriched fractions gives possibilities for development of both liquid and solid high protein foods.

8.3 Evaluation and further research

The discussed results can be divided into two research directions: (1) using a better understanding of the legume seed properties to predict the separation behaviour and (2) exploring and understanding the different functional behaviour of the fractions that are obtained. This paragraph will provide an evaluation of these results and paths for future research. Focus is put firstly on legume morphology to give insights in disentanglement of the cellular components, which is required to increase protein content, secondly on sustainability as it is the main driver for this project, and thirdly on functional properties of the fractions for application in food products.

8.3.1 Improving the separation behaviour by better understanding of the legume morphology

Protein-enrichment by air classification is based on size differences between protein-rich and protein-depleted particles in legumes. Proper milling should therefore disentangle the smaller protein bodies from the larger starch granules and cell wall fibres. Differences between starch-rich and oil-rich legumes will be discussed as well as the use of the state diagram to influence break behaviour during milling.

In both starch-rich and oil-rich legumes, separating small particles from larger ones increases the protein content of the fine fraction; however, this is accompanied by a decreased yield (Figure 51). The relation between protein content and yield depends on the initial protein content of the legume. Starch-rich legumes generally have a lower protein content than oil-rich legumes. On the contrary, the maximum protein content is similar for starch-rich and oil-rich legumes. A maximum protein content of approximately 60 g protein/100 g dry matter can be explained by the protein content inside the protein bodies, which is 70-88 g protein/100 g dry matter (Weber and Neumann, 1980; Plant and Moore, 1983), and the presence of small fibre particles in the fine fraction. For

oil-rich legumes, the maximum protein content is reached at a larger particle size than for starch-rich legumes, because the protein bodies in oil-rich legumes are larger. However, the presence of oil decreases the yield in the range of particle sizes studied. Oil reduces the dispersibility of the flour and may cause deposition of a cake layer on the walls of the air classifier.

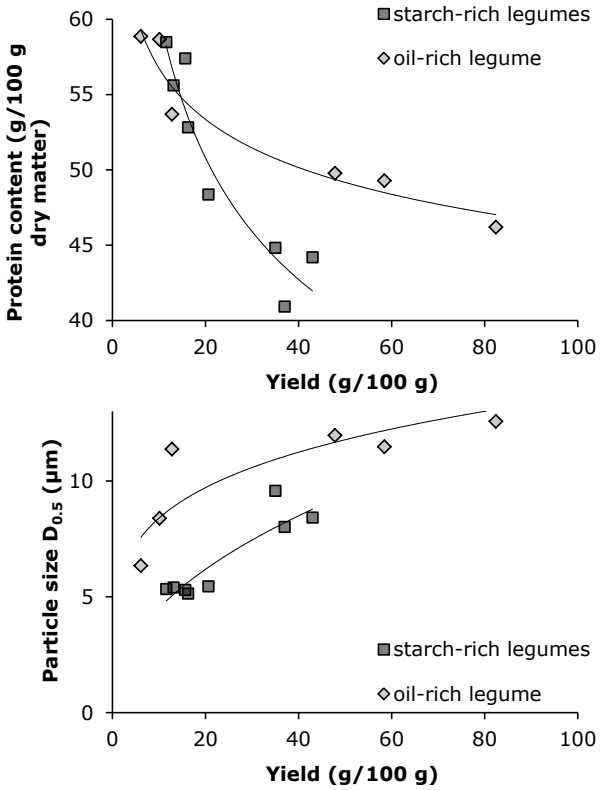


Figure 51 Relation between yield and protein content or particle size of air-classified pea and lupine fine fraction (Pelgrom, Berghout et al., 2014; Pelgrom, Boom et al., 2015a).

The protein content of the fine fraction was increased by pre-treatments like increasing moisture content or defatting of oil-rich legumes. Moisture contents were adjusted based on the hypothesis that the local fracture behaviour depends on the state, i.e. glassy or rubbery, of starch and protein. Electron scanning microscope images showed that more disentanglement takes place when protein

is in the rubbery state, which was confirmed by higher protein separation efficiencies after milling rubbery pea and lupine.

The sharpness of the transition from rubbery to glassy state is, however, debatable. The flour particle size increases gradually as a function of the moisture content as well as the protein content of particles smaller than 20 µm. Based on the glass transition curves of pea it is expected that at room temperature (20ºC) the glass transition of protein takes place at 14 g water/ 100 g dry matter and that the glass transition of starch occurs at 19 g water/100 g dry matter.

However, these values do not take into account the uneven water distribution and inhomogeneous composition in the pea seed. The GAB sorption isotherms indicated that at fixed water activity the moisture content of starch is higher than that of protein (Pelgrom, Vissers et al., 2013). When the glass transition curves of the isolates are adapted to the moisture distribution in a seed, the difference in glass transition between protein and starch becomes smaller (Figure 52). Moreover, the glass transition curve was reported to differ from the brittle-ductile transition curve for several foods, i.e. of fish meat (Watanabe, Tang et al., 1996) and gelatinized starch (Nicholls, Appleqvist et al., 1995). This could be due to a number of extrinsic factors including strain rate, stress state, specimen geometry, and presence of notches and flaws (Rahman, 2006). However, the glass transition curve of pea has been shown to coincide with ductile-brittle transitions (Pelgrom, Schutyser et al., 2013). Nevertheless, the feasibility of milling glassy starch and rubbery protein is a point of discussion.

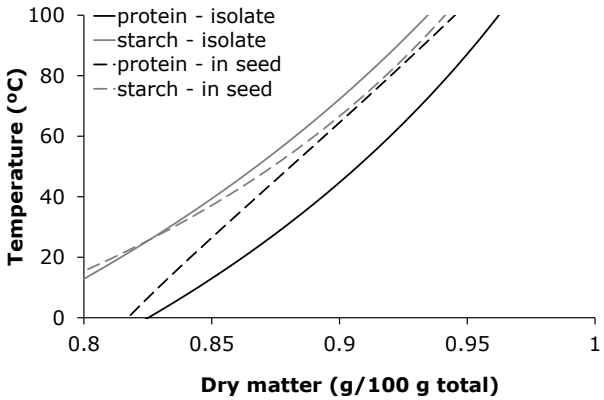


Figure 52 State diagram of pea. Solid curves are DSC results modelled with the Gordon-Taylor equation, dashed lines are adapted for the moisture distribution in the seed based on the GAB sorption isotherms (Pelgrom, Schutyser et al., 2013; Pelgrom, Vissers et al., 2013).

Further research

Better protein enrichment by dry fractionation could enable wider applicability of dry-enriched protein ingredient fractions in food industry. Although improvements were reported in this thesis, further improvement remains a challenge. One approach would be to further increase our knowledge of the exact properties of the different regions and the morphology inside legumes and relate that to even better milling. Other approaches involve the development of pre-treatments, air classification strategies or even varieties of legumes by plant breeding that better allow dry fractionation. These approaches are discussed and possibilities for further research are given.

Knowledge of legume morphology can be extended on the adhesion and hardness of fibre, protein bodies and starch granules with novel methods. The aim would be to use this information to influence break behaviour and improve disentanglement. For example, increasing the size of the fibre particles compared to protein-rich particles could reduce the fibre content in the fine fraction. Moreover, the hardness of the protein bodies and starch granules can be determined at various temperature and moisture combinations. This information will complete the state diagram and will aid in determining milling

temperature and moisture combinations that yield optimal disentanglement between the cellular components.

Atomic force microscopy (AFM), laser-induced breakdown spectroscopy (LIBS), split-Hopkinson pressure bar and advanced modelling techniques are discussed here as methods to study legume morphology. AFM is a technique to characterize locally distributed mechanical properties of flat surfaces and has been applied on wheat grains. Hard seed has been found to have different texture compared to soft seed (Scudiero and Morris, 2010). These structural differences can be related to physical–chemical differences. Another AFM study revealed that the mechanical properties of gluten resemble those of soft materials, whereas the mechanical properties of starch granules resemble that of harder materials. These mechanical properties combined with structural features are relevant to improve milling behaviour (Chichti, George et al., 2013).

The LIBS technique creates laser induced ablation craters in a sample's surface from which the physical and mechanical properties can be estimated (Singh and Thakur, 2007). Using this technique, grain milling behaviour was related to the mechanical properties of wheat tissues (Martelli, Brygo et al., 2011). The split-Hopkinson pressure bar measures stress-strain response of materials upon compression at relevant impact conditions for milling. From these data, the adhesion forces between particles can be calculated (Wanka, Kappl et al., 2013). This technique originates from metal research and could be interesting for testing legume seeds.

Particle-based modelling approaches may be used to more systematically study the parameters of influence to breakage behaviour of legumes. For example, the mechanical properties and break behaviour of wheat have been modelled with the protein matrix as a continuous phase and the starch phase being the granular phase, using a lattice-element method (Topin, Radjai et al., 2008). A toughness parameter was used to describe the starch-protein adherence and protein content.

Plant breeding and pre-treatments may lead to improved disentanglement, dispersibility or increase size differences between the cellular components. Disentanglement could be increased by selection of softer seeds. This hypothesis is based on lentil, which has a lower seed hardness compared to pea, bean and chickpea, and yielded a higher protein content in the fine fraction.

Alternatively, pre-treatments that lead to softening of the seeds could also provide easier disentanglement e.g. treatments that rupture the cellular structure but do not require water addition, like pulse electric field or freeze-drying. Softening reduces the milling intensity and thus increases the particle size of the flour, which makes it easier to disperse and to separate.

Varieties containing large starch granules could be selected to reduce the level of milling required. Additionally, for example, some chickpea cultivars contain more fibre bonds that increase adhesion between cellular components, which results in smaller particle size when attempting to liberate the starch granules during milling (Wood, Knights et al., 2014). In this case the fibres will be milled too fine and will enter the fine fraction. Therefore one may select cultivars that contain tougher fibre or specifically degrade the fibres with chemicals such as sulphur dioxide, sodium hydroxide or sulphuric acid. The use of these chemicals may however degrade the material and will compromise the sustainability of the fractionation process.

Finally, further development of air classifiers is desirable to provide a sharper separation between the coarse and the fine fractions. The classifier wheel house can be redesigned to reduce the amount of material that is blocked there and hinders separation. A suggestion would be to develop a round house containing a rotating wall scraper. Next to that, the angle and the design of the vanes could be optimized for legume flour (Huang, Liu et al., 2012).

8.3.2 Sustainability

Renewed interest into dry fractionation of plant protein is amongst others motivated by its lower energy and water use compared to wet fractionation processes. This is confirmed by estimations of the energy and water use per kg

end product (Figure 53). Even though industrial dry fractionation facilities exist with typically a capacity of 90 000 tons per year, wet fractionation is the mainstream technology for plant protein extraction. Wet fractionation of starch-rich legumes typically starts by diluting flour to a suspension of 13 g flour/100 g solution. A second dilution step is carried out before spray drying (Figure 53) (Passe, Fouache et al., 2008). These two dilution steps result in a consumption of 50 kg water/kg recovered protein. For oil-rich legumes, water consumption of 90 kg water/kg protein has been reported (Berghout, Pelgrom et al., 2015). In contrast, dry fractionation by definition consumes no water. Part of the added water during wet fractionation is removed by spray drying, which is the main cause for the difference in energy use between dry (3.6 MJ/kg recovered protein) and wet (54 MJ/kg recovered protein) fractionation.

Wet fractionation of plant protein still consumes much less water compared to the production of animal protein. It is estimated that primary production of 1 kg of animal protein requires about 200 m^3 water compared to the water use of 0.5 – 2 m^3 for the cultivation of 1 kg of cereal protein (Pimentel and Pimentel, 2003). The water required to extract proteins is much less, approximately 2.5 to 20% of the water use of the cultivation. The protein delivery efficiency was calculated as the ratio of protein to invested life cycle energy in foods and showed that animal products can provide 4-11 g protein per MJ whereas legumes can provide 41-77 g protein per MJ (González, Frostell et al., 2011). It can be concluded that the protein delivery efficiency is much larger for legume-based foods compared to animal-based foods. When protein extraction is taken into account, the ratio for wet-extracted pea protein decreases to 14.6 g protein per MJ, but for dry-fractionated pea, it remains 55.8 g protein per MJ. The latter calculation shows that the ratio for wet-extracted protein is nearly equal to that of animal foods, which emphasizes the importance to explore the more efficient dry fractionation.

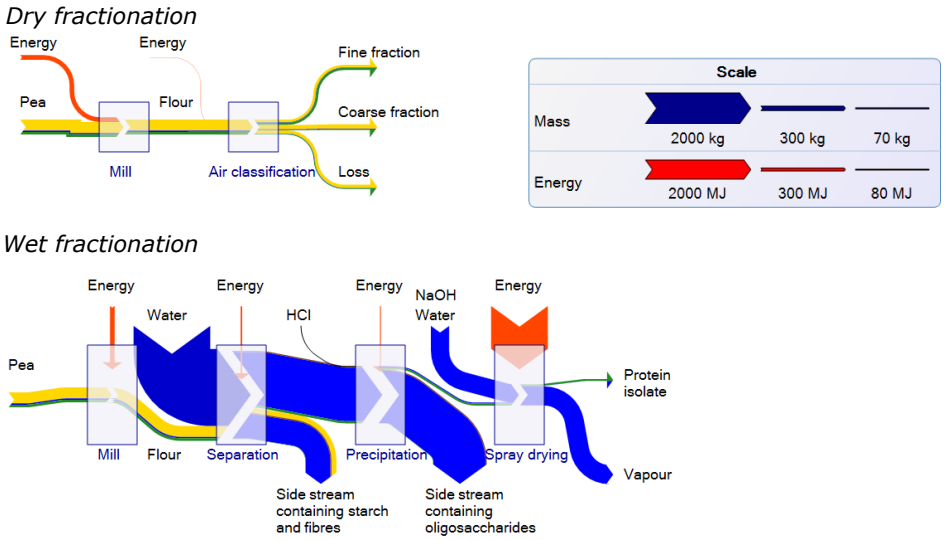


Figure 53 Sankey diagrams of dry fractionation (left) based on (Pelgrom, Boom et al., 2015a) and wet fractionation (right) based on (Passe, Fouache et al., 2008). Process streams containing water (blue), protein (green), starch, fibre, oil and ash (yellow) and energy (orange) are depicted. For simplicity reasons mass flows and energy are given in the same diagram.

Further research

Dry fractionation is a more sustainable process in terms of water and energy use compared to wet fractionation; however the protein purity of the dry-enriched fractions is lower than that of the wet-enriched fractions.

In this thesis a hybrid process involving successive dry and aqueous fractionation was introduced to obtain a higher protein content, which requires less water and energy than wet fractionation only (Pelgrom, Boom et al., 2015b). The differences between conventional wet fractionation and the new hybrid process are that suspension of flour takes place at a lower dilution, no chemicals (HCl, NaOH) are used and no dilution takes place before spray drying. The energy and water use of this combined process were graphically represented per kg product in Figure 54. The fine fraction obtained by dry fractionation was suspended in water and the purified by a centrifugation and a filtration step.

Scaling-up of the lab centrifuge and Amicon ultrafiltration cell can be realised by using for example a conventional centrifugal decanter and a spiral-wound ultrafiltration membrane system. The water consumption was reduced to 13 kg/kg protein because less powder had to be suspended; the fine fraction was 35% of the total amount of flour, but contained 81% of the proteins present in the flour. Reducing the water consumption by combining dry and aqueous fractionation is not specific for pea, but has also been proposed for lupine (Berghout, Pelgrom et al., 2015). The energy consumption can be reduced to 20 MJ per kg recovered pea protein, mainly because no water is added before spray drying. This water addition is needed in the conventional wet fractionation process to reduce the viscosity (17.8 ± 2.2 Pa s at 25ºC, shear rate of $100s^{-1}$ and 30 g solids/100 g sample) (Pawlowski, 2008; Bouvier and Campanella, 2014). The viscosity of the protein solution obtained with the hybrid process is lower at similar conditions (0.3 ± 0.0 Pa s at 25ºC, shear rate of $100s^{-1}$ and 30 g solids/100 g sample) because the proteins are in the native state (Pelgrom, Boom et al., 2015b).

The energy consumption calculations do not take into account any further utilisation of side streams. The side streams of wet fractionation contain more water and therefore require more energy to be processed into products. In general, side streams should be avoided when no direct product application is possible.

Overall, utilisation of side streams will make processes more sustainable (Zisopoulos, Rossier-Miranda et al., 2014). The coarse fraction is rich in starch and is therefore valuable for many applications (Gómez, Doyagüe et al., 2012). Fibre-rich side streams could be used as additives to health promoting foods and beverages (Dalgetty and Baik, 2003). Next to that, side streams can be used for non-food applications like: food packaging material (Mikkonen and Tenkanen, 2012), bioethanol, biogas or animal feed (Draganovic, Goot et al., 2013). In conclusion, the combination of dry and wet fractionation could be explored further for possible scale-up as the protein purity and yield are comparable to the traditional wet fractionation process but at reduced water consumption.

Dry fractionation combined with aqueous fractionation

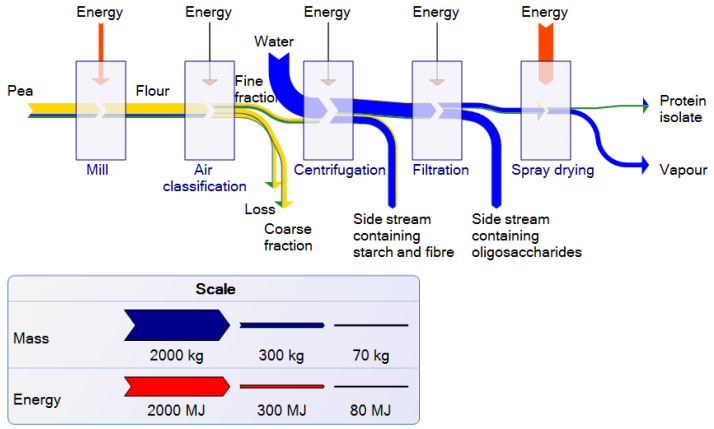


Figure 54 Sankey diagrams of the combined dry and aqueous fractionation process based on (Pelgrom, Boom et al., 2015b). Process streams containing water (blue), protein (green), starch, fibre, oil and ash (yellow) and energy (orange) are depicted. For simplicity reasons mass flows and energy are given in the same diagram.

Table 17 Energy use per tonne processed material of various process steps used in dry, aqueous or wet fractionation. An energy efficiency factor of 0.5 was used for heating and drying processes. Cooling and transport energies are not taken into account.

Process step	Energy (MJ/tonne)	Reference
Mill	500	(Schutyser and Van der Goot, 2011)
Air classification	23	(Schutyser and Van der Goot, 2011)
Centrifugal decanter/ hydrocyclone	15	(Grimwood, 2011; Haverinen, 2014)
Nozzle	6	(Hui, 2008)
Spray dryer	4800*	(Schutyser and Van der Goot, 2011)
Ultrafiltration	14	(Cheryan and Kuo, 1984; Ramirez, Patel et al., 2006)

** for spray drying per tonne of evaporated water*

8.3.3 Functionality

Dry fractionation yields protein concentrates that exhibit native properties, but at a lower protein purity compared to wet-fractionated isolates. The native properties are reflected in better solubility, foam stability, digestibility and lower viscosity compared to conventional protein isolates (Table 18). However, differences are observed between functional properties of various legumes. The viscosity of the fine fraction of lupine is lower than that of the fine fraction of pea, but the viscosity of lupine flour is higher than that of pea flour (Pelgrom, Vissers et al., 2013; Pelgrom, Berghout et al., 2014). These differences can be explained by the higher solubility of lupine protein and the higher amount of water absorbing fibres in lupine flour. Moreover, lupine protein forms very weak heat-induced gels, while denatured pea protein can form strong heat-induced gels (Berghout, Boom et al., 2014b; Pelgrom, Boom et al., 2015b).

Applications of protein concentrates can thus be found in products that require high solubility, like beverages (high protein drinks) or products in which emulsification, water and fat absorption and adhesive properties are required, like baked goods, pasta, granola bars, meat products, vegetarian burgers and texturized products (Tulbek, 2010). Protein concentrate applications overlap with the applications of protein isolates (RoquetteFreres, 2008; Boye, Zare et al., 2010). Next to the lower water and energy use in production, protein concentrates are associated with health benefits compared to further refined ingredients (Jacobs, Gross et al., 2009). Therefore, one may move from pure protein isolates to less refined concentrates, such as the ones investigated in this thesis. This would result in ingredients that require far less resources to produce, while at the same time providing better composition for our health (fibre, micronutrients). Challenges that accompany this transition would be in new product formulations, more variability in the ingredients and differences in taste and nutritional value; yet, it would also generate new freedom in these fields, for new types and qualities of products.

Table 18 Functional properties of the various fractions obtained by air classification and wet fractionation (Pelgrom, Vissers et al., 2013; Pelgrom, Berghout et al., 2014; Pelgrom, Boom et al., 2015b).

	Coarse	Fine	Protein isolate
Starch-rich legumes (pea)	Low solubility Heat-induced gelation	High solubility Enzymatic gelation	Lower solubility Enzymatic gelation
Oil-rich legumes (lupine)		Foam stability Low viscosity More peptides after digestion	Lower foam stability Higher viscosity Less peptides after digestion

Differences in taste and nutritional value originate from nutritionally active components that are partly deactivated during wet fractionation, but not during dry fractionation. These components are: protease inhibitors (trypsin inhibitors), amylase inhibitors, lectins, polyphenols, saponin and phytic acid (Asgar, Fazilah et al., 2010). Some of these components may influence the uptake of nutrients during digestion (Elkowicz and Sosulski, 1982; Guillamon, Pedrosa et al., 2008; Schlemmer, Frølich et al., 2009).

Recently, they have also been associated with health promoting properties, like anti-oxidant and anti-carcinogenic activity (Shi, Arunasalam et al., 2004; Guillamon, Pedrosa et al., 2008; Schlemmer, Frølich et al., 2009). Toasting is frequently applied to treat dry-enriched ingredient fractions to remove their bitter or astringent taste, for which saponins are responsible (Curl, Price et al., 1985). Upon post-processing, like cooking, protease inhibitors, amylase inhibitors, lectins and polyphenols are deactivated (Trugo, Donangelo et al., 2000; Asgar, Fazilah et al., 2010), and the amount of saponin is reduced by 7 to 53% (Shi, Arunasalam et al., 2004), whereas phytic acid is heat stable (Trugo, Donangelo et al., 2000; Schlemmer, Frølich et al., 2009).

Air-classified ingredient fractions usually also contain lipoxygenase providing a beany flavour to legumes. This enzyme may be deactivated during mild heating at 60ºC, thus without having a detrimental effect to the degree of protein denaturation (Asgar, Fazilah et al., 2010). Additional research could further balance the functionality, nutritional value and taste of the dry ingredient fractions.

Further research

In this thesis, pea fractions have shown to form a gel under non-flow conditions and to phase separate upon suspension in water (Pelgrom, Boom et al., 2015b). This means that the pea fraction possesses the two main properties needed to form aligned structures by gelation under shear-flow conditions (Manski, van der Goot et al., 2007). Aligned structures are characterised by a stronger structure in the direction parallel to the shear than in the direction perpendicular to the shear. Further research into structure formation may increase the range of applications.

All pea fractions formed aligned structures in a shear cell (Figure 55), indicating that components of pea (starch, protein and fibres) align in the direction of the shear. This finding is not trivial. Earlier research concluded that suspensions of wheat starch and corn zein only orient along the flow at low concentrations of zein and at high shear speeds (Habeych, Dekkers et al., 2008). Moreover, wheat dough separated under shear in gluten patches and a starch-rich phase (Peighambardoust, Hamer et al., 2008).

Even though the ratio of the material strengths parallel and perpendicular to the shearing direction was similar for the coarse fraction and the fine fraction using transglutaminase as a cross-linking additive, the latter formed a layered structure whereas the coarse fraction gave no layers. Commercial pea protein isolates formed a structure with the highest ratio, which was in agreement with the layered structure visually observed (Figure 55). For comparison, the ratio in chicken filet is 1.7 and the ratio in hamburgers is close to 1. Structure formation using pea protein was also observed after high moisture extrusion cooking (Osen, Toelstede et al., 2014). In conclusion, there is potential for the formation

of aligned structures, like meat replacers, from dry-enriched pea fractions, which justifies further research in this field.

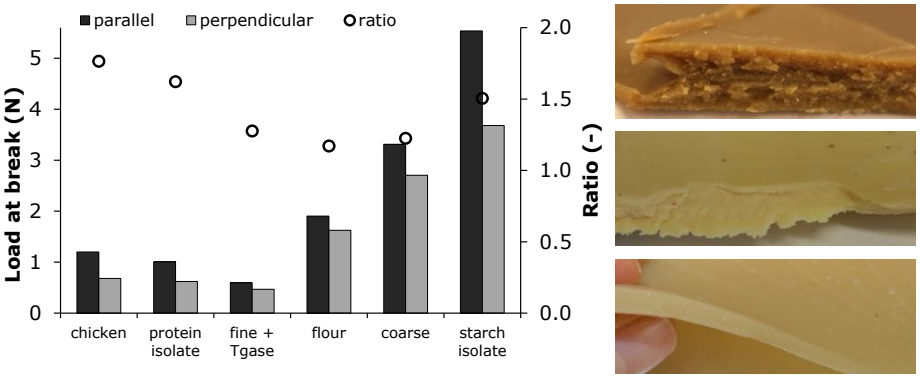


Figure 55 Strength of structures formed in the shear cell (140ºC, 15 min, 30rpm or with transglutaminase 50ºC, 35 min, 30rpm) from various pea fractions (44 g/g sample, 1 g/g salt and 55 g/g tap water, and to the fine fraction transglutaminase was added to obtain an enzyme to protein ratio of 1:20). Load at break was determined using a Texture Analyser. Samples were cut to a bog-bone-shape in the direction parallel and perpendicular to the shear or for chicken breast filet parallel and perpendicular to the direction of the meat fibre. Images on the right represent protein isolate, fine fraction with transglutaminase and coarse fraction.

8.4 Summary of key findings

This thesis has contributed to the awareness that the food industry could exploit a more sustainable dry fractionation technique to obtain functional protein fractions rather than focussing on wet extraction of relatively pure protein ingredients.

Functional fractions were obtained by selecting dedicated milling settings for starch-rich and oil-rich legumes based on legume morphology. Milling settings were estimated based on starch granule size in starch-rich legumes, whereas coarse milling provided better results for oil-rich legumes. Separation of the protein bodies from other cellular components was established by air classification, which consumed ten times as little energy as conventional wet fractionation and 50 litres of water per kg protein less.

The dry-enriched fractions possessed higher solubility than conventionally produced fractions, making them suitable for high protein drinks. Pea fractions could also be gelatinized, which opens opportunities for preparing solid protein foods.

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Summary

Our food production system should become increasingly more sustainable to cope with the growing world population while living on a planet with limited natural resources. Transition from animal to plant protein sourcing for food production could be part of the solution since 1 kg of animal protein requires 6 kg of plant protein feed. In particular, legumes, such as pea, lupine, bean, chickpea and lentil, are of interest because of their high protein delivery efficiency, which is approximately 8 times as much as animal products in terms of g protein per MJ invested energy. In addition, the traditional energy-consuming wet legume protein isolation processes could be replaced by much more efficient dry fractionation processes. Dry fractionation of legumes is based on tissue morphology and involves dry milling and separation of starch granules and protein bodies based on their difference in size. Another major advantage is that dry fractionation retains native protein functionality as it does not use pH shifts or elevated temperatures. Moreover, dry-enriched legume proteins are low in fat and have an excellent nutritional profile, being rich in protein, dietary fibre and a variety of micronutrients and phytochemicals. A possible drawback is that the purity of the dry-enriched proteins is lower (55 g protein/100 g dry matter) than that of wet-enriched proteins (80 g protein/100 g dry matter). However, recent insights suggest that functionality of dry-enriched fractions should be valued rather than their protein purity. Those insights together with the need for a more sustainable food supply have renewed the interest in dry fractionation. Despite advances in dry separation in the past, gaps in our knowledge of dry fractionation were identified, such as the relation between seed morphology and optimal disentanglement during milling and the difference in functionality between dry- and wet-enriched protein fractions. Therefore, the objectives of this thesis were to understand material properties of legume seeds better to find more optimal milling and air classification conditions and to analyse functionality of these sustainably-produced fractions.

This thesis starts with a systematic study of milling and air classification of pea (**Chapter 2**). Pea seeds contain starch granules (± 20 µm) that are embedded

in a matrix of protein bodies (1-3 μm) which is surrounded by a fibre-rich cell wall. Milling should disentangle these cellular components so that protein- and starch-rich particles can be separated during air classification based on size and density. Milling was found to have an optimum: too coarse a milling gave insufficient disentanglement between the cellular components and too fine a milling yielded similar size for all components. Subsequent air classification yielded protein concentrates containing 55 g protein/100 g dry matter. At this protein content, small particles (5 μm) entered the fine fraction causing build-up of material in the classifier due to increased van der Waals force interactions. The functionality of the fraction was evaluated with water holding capacity (WHC) tests. Dry-enriched pea protein showed high solubility and pea starch formed heat-induced gels.

Disentanglement of protein bodies and starch granules in pea seeds was analysed in more detail as function of moisture content and temperature (**Chapter 3**). Glass transition curves were constructed based on differential scanning calorimetry (DSC) and thermal mechanical compression tests (TMCT) data using the Gordon-Taylor equation. Peas were brought in the glassy or rubbery state and breakage force and fracture plane were analysed. Glassy peas broke at lower energy input and broke along a flat fracture plane. Rubbery peas required higher energy input, but the fracture plane showed more disentanglement. This suggested that more disentanglement takes place in rubbery peas upon breakage.

The functionality of dry-enriched pea fractions was further explored in terms of phase separation and gelatinization (**Chapter 4**). Upon suspension in water, dry-enriched pea fraction separated into four distinctive layers. The two top layers were concentrated by ultrafiltration into a native protein concentrate with a purity of 77 g protein/100 g dry matter and a similar protein yield as wet fractionation (63%). This combination of dry and aqueous fractionation consumed less water and energy compared to wet fractionation and was therefore proposed as a more sustainable route to obtain pea protein isolates. Besides, starch-enriched fractions were gelatinized by heat and protein-enriched fractions were gelatinized by enzymatic crosslinking. The enzymatic cross-linked

dry-enriched protein gels were firmer than wet-enriched protein gels at similar concentration due to the presence of wate-absorbing fibre. This result suggests that development of novel food ingredients should focus on functionality rather than on molecular purity.

Dry fractionation was evaluated for oil-rich legumes, i.e. lupine (**Chapter 5**). Lupine seeds contain protein bodies (5-25 µm) that are surrounded by a fibre-rich cell wall. Dry fractionation was found more optimal when lupine was milled more coarse compared to pea. Coarse milling disentangled protein bodies and avoided strong size reduction of fibres. Protein concentrates with 59 g protein/100 g dry matter were obtained. Yields were low due to 7 g fat/100 g dry matter, which caused build-up of material in the classifier, but could be doubled by addition of flowability aids. Functional analyses of lupine protein concentrates showed that dry enriched lupine proteins had a higher foam stability, digestibility and lower viscosity compared to denatured wet-enriched proteins.

The protein purity and yield of pea and lupine achieved with dry fractionation was increased by applying pre- and post-treatments (**Chapter 6**). Pre-treatment that led to increased moisture content brought legumes in the rubbery state. This transition increased disentanglement during milling, which was confirmed by a higher protein content of small (<20 µm) particles. Defatting was also successfully applied as it increased protein purity and yield for lupine due to improved flowability. The post-treatment consisted of electrostatic separation and increased the protein content of fine and coarse fractions. Protein and starch charged positively and could thus be separated from fibre, which charged more negatively.

Dry fractionation results of pea were used to develop the hypothesis that, for starch-rich legumes, optimal disentanglement would be obtained when the particle size distribution curve of flour and isolated starch granules maximally overlapped (**Chapter 7**). This hypothesis was found valid for pea, bean, lentil and chickpea. Milling settings, to reach maximal overlap, were dependent on seed hardness and starch granule size. The protein content of the fine fraction

was legume specific as well, which were related to differences in particle density, starch granule size, fat content and flour dispersibility.

Finally, the results of this thesis were discussed and an outlook for further research was given around the three major themes of this thesis, i.e. increasing knowledge of legume morphology related to dry fractionation, sustainability of protein extraction from legumes and functionality of dry enriched fractions (**Chapter 8**). Differences in legume morphology between starch-rich and oil-rich legumes and the consequences for dry fractionation were discussed as well as the translation of glass transition curves to the state of components in the seed. Sustainability analyses were visualised in Sankey diagrams and provided numbers on water and energy use, i.e. for dry fractionation (0 kg water/kg protein, 3.6 MJ/kg protein), for conventional wet extraction (50 kg/kg protein, 54 MJ/kg protein) and the combined dry and aqueous processing (13 kg water/kg protein, 20 MJ/kg protein). Finally, possible applications for dry-enriched fractions were discussed and the possibility to prepare meat replacers was evaluated. It was concluded that dry fractionation of legumes is much more efficient while delivering highly functional protein fractions and could thus be part of the solution towards a more sustainable food production system.

Samenvatting

De alsmaar groeiende wereldbevolking en de beperkte middelen op aarde vragen om een duurzamere productie van voedsel. De omschakeling naar een dieet met meer plantaardige eiwit in plaats van dierlijke eiwit kan zowel de voedselproductie verhogen als de negatieve invloed daarvan op ons milieu verkleinen, omdat voor de productie van 1 kg dierlijk eiwit ongeveer 6 kg plantaardig eiwit nodig is. Peulvruchten, zoals erwten, lupine, witte bonen, kikkererwten en linzen, zijn naast hun hoge nutritionele waarde, erg interessant vanwege hun acht keer hogere eiwit productie efficiëntie uitgedrukt in gram eiwit per megajoule gebruikte hoeveelheid energie in vergelijking met dierlijke producten. Traditionele natte scheidingsprocessen voor de isolatie van plantaardige eiwitten hebben een hoog water- en energieverbruik. Dit kan flink gereduceerd worden als gebruik wordt gemaakt van droge fractionering door middel van droogmalen en luchtclassificatie.

Droge fractionering maakt gebruik van de natuurlijke opbouw van de zaadlobcellen in peulvruchten. Na fijn malen valt deze structuur uiteen en kunnen de grotere zetmeelkorrels gescheiden worden van kleinere eiwitrijke fragmenten. Een groot voordeel van droge fractionering is dat geen pH veranderingen en geen hoge temperaturen tijdens het proces voorkomen, waardoor natieve eigenschappen van de eiwitten behouden blijven. Naast een hoog eiwitgehalte en een laag vetgehalte, zijn de droge eiwitconcentraten rijk aan vezels en verschillende micronutriënten en fytochemicaliën. Een nadeel van droog fractioneren is dat het eiwitgehalte van de verrijkte fracties lager is (55 g eiwit / 100 g droge stof) dan dat van nat verrijkte fracties (80 g eiwit / 100 g droge stof). Echter, nieuwe inzichten laten zien dat technologische eigenschappen van droog verrijkte fracties minstens even belangrijk, zo niet belangrijker, zijn als moleculaire zuiverheid. Dit inzicht, samen met de behoefte aan een duurzamere productie van voedsel heeft de interesse in droog fractioneren weer doen groeien.

Ondanks bestaande inzichten over droog fractioneren zijn veel vragen onbeantwoord. Bijvoorbeeld, wat de relatie is tussen morfologische

eigenschappen van peulvruchten en maalgedrag dat leidt tot optimale ontsluiting van de eiwitten en wat de invloed is van het droog fractioneren op de eigenschappen van de droog verrijkte eiwit fracties ten opzichte van de traditionele eiwit concentraten. De doelen van het onderzoek beschreven in dit proefschrift waren dan ook om de materiaaleigenschappen van peulvruchten beter te begrijpen en daarmee optimale maal- en scheidingscondities te bepalen en tot slot de functionele eigenschappen van deze fracties te evalueren.

Dit proefschrift begint met een systematische studie naar malen en luchtclassificatie van erwten (**Hoofdstuk 2**). Erwten bevatten zetmeelkorrels (± 20 µm) ingesloten in een matrix van eiwitorganellen (1-3 µm) met daarom heen een vezelrijke celwand. Het doel van malen is om deze componenten fysiek van elkaar los te maken, zodat met luchtclassificatie de eiwit- en zetmeelrijke deeltjes kunnen worden gescheiden op basis van grootte en dichtheid. Er is een optimum voor de deeltjesgrootte na het malen: te grof malen geeft onvoldoende ontsluiting van de componenten en te fijn malen leidt tot identieke grootte van de componenten. Na optimalisatie van het maalproces malen kan met luchtclassificatie een erwten eiwitconcentraat gemaakt worden met 55 g eiwit/100 gram droge stof. Het eiwitconcentraat wordt gewonnen als de fijne fractie en bestaat uit kleine deeltjes (~5 µm diameter) waarmee het risico bestaat voor vervuiling in het roterende classificatie wiel. De functionaliteit van de fracties is geëvalueerd met hulp van het waterhoudend vermogen, waaruit bleek dat de droog verrijkte eiwitten fractie bijzonder goed oplosbaar zijn. Daarnaast is gevonden dat de aanwezigheid van zetmeel helpt bij het vormen van gels na verwarmen.

De hypothese was vervolgens dat verschillende mechanische eigenschappen van zetmeel en eiwit een beter breukgedrag zouden kunnen geven. Deze eigenschappen kunnen gestuurd worden met vochtgehalte en temperatuur (**Hoofdstuk 3**). Hiervoor zijn glastemperatuur curves bepaald met differentiële scanning calorimetrie en thermisch mechanische compressie testen en beschreven met de Gordon-Taylor-vergelijking. Vervolgens zijn erwten onder verschillende condities gebroken en zijn kracht en breukoppervlakte geanalyseerd. Het bleek dat erwten in glastoestand makkelijker breken en dat

het breukoppervlak vrij vlak is, terwijl in rubbertoestand meer kracht nodig is voor breuk en het breukoppervlak onregelmatiger is rond de zetmeelkorrels. Dit suggereert dat de zetmeelkorrels beter ontsloten worden in een rubbertoestand.

De functionaliteit van droog verrijkte erwten fracties werd verder onderzocht op waterige fasescheiding en gelering (**Hoofdstuk 4**). Suspenderen en centrifugatie van de natieve fracties resulteerde in fasescheiding in vier lagen met verschillende compositie. De twee bovenste lagen konden gedecanteerd en geconcentreerd worden door middel van ultrafiltratie tot een eiwitconcentraat met 77 g eiwit/100 g droge stof met een vergelijkbare eiwitopbrengst als in het traditioneel natte scheidingsproces (63%). Deze combinatie van droog scheiden en waterige fase scheiding verbruikt minder water en energie is dus duurzamer dan het natte scheidingsproces. Daarnaast werden gels gemaakt van zetmeelrijke fracties door middel van verwarming en van eiwitrijke fracties door middel van enzymatische verbindingen. Het bleek dat droog verrijkt eiwit sterkere gels geeft dan eiwit-isolaten mits geëvalueerd bij dezelfde concentratie, waarschijnlijk als gevolg van de aanwezigheid van water absorberende vezels. Dit resultaat toont aan dat het meerwaarde heeft om bij de ontwikkeling van ingrediënten te focussen op functionaliteit in plaats van op moleculaire zuiverheid.

Naast droge fractionering van erwten is ook lupine onderzocht, een olierijke peulvrucht (**Hoofdstuk 5**). Lupinezaden bestaan uit eiwitorganellen (5-25 µm) met daaromheen een vezelrijke celwand. Droog fractioneren van grof gemalen lupine is optimaler, aangezien op deze manier de eiwitorganellen fysiek gescheiden worden en de vezels niet te klein worden vermalen tot eenzelfde grootte. Luchtclassificatie na malen leverde lupine eiwitconcentraten met 59 g eiwit/100 g droge stof. Het relatief hoge vetgehalte van lupine (7 g vet/100 g droge stof) heeft een negatieve invloed op de opbrengst, maar deze kon verhoogd worden door toevoeging van een antiklontermiddel. Tot slot werd gevonden dat droog verrijkte lupine eiwitten een hogere schuimstabiliteit, een beter verteerbaarheid en een lagere viscositeit hebben in vergelijking met verwarmde en daardoor deels gedenatureerde eiwitten.

Om de eiwitzuiverheid en opbrengst van erwt en lupine na droog fractioneren verder te verhogen kan dit proces mogelijk gecombineerd worden met voor- en nabehandelingen (**Hoofdstuk 6**). Zoals eerder besproken kunnen de mechanische eigenschappen en uiteindelijke breuk met behulp van water en temperatuur gestuurd worden. Uit maal- en scheidingsexperimenten bleek dat erwten in de rubbertoestand inderdaad leidde tot een hoger eiwitgehalte van de kleine deeltjes (<20 µm). Daarnaast is gevonden dat ontvetten voor malen de eiwitzuiverheid en de opbrengst voor lupine vergroot, waarschijnlijk door betere dispergeerbaarheid van de lupinebloem. Nabehandeling met behulp van elektrostatisch scheiden blijkt ook het eiwitgehalte van de fijne en grove fracties van lupine verder te kunnen verhogen. Eiwit en zetmeeldeeltjes worden positief geladen en kunnen daardoor worden gescheiden van vezels die negatief geladen worden.

Naar aanleiding van de eerdere droge scheidingsresultaten van erwten is een hypothese opgesteld voor zetmeelrijke peulvruchten om op een snelle manier optimale maalcondities vast te stellen voor dit type peulvruchten. De hypothese stelt dat optimale ontsluiting wordt verkregen als de deeltjesgrootte verdeling van de bloem en die van de zetmeelkorrels maximaal overlappen (**Hoofdstuk 7**). Deze hypothese is geëvalueerd voor erwten, bonen, linzen en kikkererwten, waarbij bleek dat maalinstellingen om maximale overlap te bereiken afhankelijk zijn van zaadhardheid en zetmeelkorrelgrootte. Verder is gevonden dat het uiteindelijke eiwitgehalte van de fijne fractie specifiek is voor iedere peulvrucht en afhangt van deeltjesdichtheid, zetmeelkorrelgrootte, vetgehalte en dispergeerbaarheid de bloem.

In het laatste hoofdstuk is een synthese gemaakt van de resultaten van dit proefschrift. Verder zijn vooruitzichten voor verder onderzoek geformuleerd rond de drie thema's in dit proefschrift: inzicht in peulvrucht morfologie in relatie tot breukgedrag, duurzaamheidsaspecten van droge fractionering van peulvruchten en functionaliteit van de verrijkte fracties (**Hoofdstuk 8**). De voornaamste verschillen in morfologie tussen zetmeelrijke en olierijke peulvruchten en de gevolgen voor droog fractioneren worden besproken, evenals de toepasbaarheid van glastemperatuur op breukgedrag. Het water- en energie verbruik van de

verschillende eiwit extractieroutes is geanalyseerd en gevisualiseerd met Sankey-diagrammen. Grote verschillen in water en energiegebruik tussen de extractieroutes zijn gevonden: droog fractioneren (0 kg water / kg eiwit, 3,6 MJ / kg eiwit), nat fractioneren (50 kg / kg eiwit, 54 MJ / kg eiwit) en het gecombineerde droge en waterige scheidingsproces (13 kg water / kg eiwit, 20 MJ / kg eiwit). Tot slot zijn de toepassingsmogelijkheden voor droog verrijkte fracties bediscussieerd waarbij eerste resultaten worden getoond om vleesvervangers te maken. De conclusie is dat droog fractioneren van peulvruchten efficiënter is en eiwitfracties geeft met zeer interessante technologische eigenschappen. Daarmee draagt dit werk bij aan de oplossing van een duurzamere voedselproductie.

Acknowledgements

In this section, I would like to thank everybody who contributed to this thesis. First, I would like to acknowledge the PEAS Foundation for their financial support and ISPT for project coordination support through the Intensified Protein Structuring project.

I would like to thank Maarten, my daily supervisor, for our pleasant collaboration. Our weekly meetings always made me feel more positive about my work. Thank you for teaching me how to write scientific papers, for putting me back on the helicopter when I lost the overview and for correcting my papers so quickly. Remko, thank you for being my promotor. Our meetings gave me a lot of new ideas and insights. Many thanks also to Atze Jan, our project leader, and the PhD colleagues of the Intensified Protein Structuring project, Jacqueline, Kasia and George, for our great collaboration.

Next, I would like to thank my colleagues at the Food Process Engineering department. Big thanks to the technicians for helping me in the lab and to the secretaries, Joyce and Marjan, for organizing everything smoothly. Thank you to my students, Desiree, Anne, Memet Ali, Christa, Ying and Li, for all the work that they have done. And to all my colleagues, thank you for the relaxing breaks and talks.

Besides, I'm grateful to my friends for their support outside work. A million thanks to my study friends (Nicole, Jacqueline, Milou, Jacob, Marieke, Thomas and Inge), my flatmates (Marlinda, Erik and Guido), the promco (Rolf and Joost) and the W10 moppies (Vera, Moyo, Sjak, Leo and Lies).

Tot slot bedank ik Mama, Papa, Alexandra en de rest van de familie voor jullie interesse in mijn werk en jullie hulp daarbij. Je voudrais remercier ma belle-famille pour les weekends et semaines de détente et pour votre gentillesse. Et Cyril, merci beaucoup that you er altijd voor me bent.

About the author

Pascalle Pelgrom was born in Eindhoven (The Netherlands) on January 7th 1987. She finished her secondary education (Gymnasium) in 2005 at Carolus Borromeus College in Helmond. That same year she started with the study Food Technology at Wageningen University. She finished her BSc in 2008 and wrote her thesis on "Optimizing the level of lycopene in watermelon salad" at the department of Food Quality and Design, in collaboration with Fruitmasters and TNO. Subsequently, Pascalle chose the MSc specialization "Process and product design". During her MSc thesis, she studied "Structuring of gluten free dough" at the department of Food Process Engineering. For her internship Pascalle went to New Zealand, where she worked at the R&D department of Fonterra Research Centre.

In 2010, Pascalle obtained her MSc degree and started her PhD at the department of Food Process Engineering. She studied dry fractionation of legumes to obtain sustainable and functional protein concentrates. The results from these four years of research are described in this thesis. Since January 2015, Pascalle works at Everris as Process Development Engineer on coated fertilizers for grass, plants and crops.

Publications

Schutyser, M.A.I., Pelgrom, P.J.M, van der Goot, A.J., Boom, R.M. (2015) Dry fractionation for sustainable production of functional legume protein concentrates. *Submitted to journal.*

Pelgrom, P.J.M., Boom, R.M., Schutyser, M.A.I. (2015) Dry fractionation of starch-rich legumes. *Submitted to journal.*

Pelgrom, P.J.M., Boom, R.M., Schutyser, M.A.I. (2015) Pre- and post-treatment enhance the protein enrichment from milling and air classification of legumes. *Journal of Food Engineering.*

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van Riemsdijk, L.E., Pelgrom, P.J.M., van der Goot, A.J., Boom, R.M., Hamer, R.J. (2011). A novel method to prepare gluten-free dough using a meso-structured whey protein particle system. *Journal of cereal science, 53* (1), 133-138.

Overview of completed training activities

Discipline specific activities

Food perception and food preference, The Netherlands	2011
Sustainability Analysis in Food Production, The Netherlands	2011
Granulation Course, United Kingdom	2011
Netherlands Process Technology Symposium, The Netherlands	2011
Dagsymposium drogen, The Netherlands	2011
International Symposium on Food Rheology and Structure, Swiss (presentation)	2012
Biorefinery for Biomolecules, The Netherlands	2012
Rheology and structure of food, The Netherlands	2012
Hosokawa AlpineExpo, Germany	2012
International Conference for Conveying and Handling of Particular Solids, Germany (presentation)	2012
European Federation of Food Science & Technology Conference, France	2012
Biorefinery for Food, Fuel and Materials, The Netherlands (presentation)	2013
European Congress of Chemical Engineering, The Netherlands	2013
New developments in characterization of Dispersions and Powders, The Netherlands	2013
From Model Foods to Food Models, France	2013
Solids, The Netherlands (presentation)	2013
Microscopy course, The Netherlands	2013
Netherlands Process Technology Symposium, The Netherlands (presentation)	2014

General courses / activities

Working in projects, The Netherlands	2011
PhD VLAG week, The Netherlands	2011
Competence Assessment, The Netherlands	2011
Afstudeervak begeleiden, The Netherlands	2011
Interpersonal Communication for PhD Students, The Netherlands	2011
Scientific writing, The Netherlands	2012
Presentation Skills, The Netherlands	2012

Optional activities

Food Process Engineering Group day	2011-2014
Food Process Engineering study tour, Finland & Baltic States	2012
Food Process Engineering study tour, Chili & Brazil	2014

The work presented in this thesis has been carried out in the framework of the Institute for Sustainable Process Technology (ISPT) under the project "PI-00-03 IPS: Intensified Protein Structuring for More Sustainable Food".