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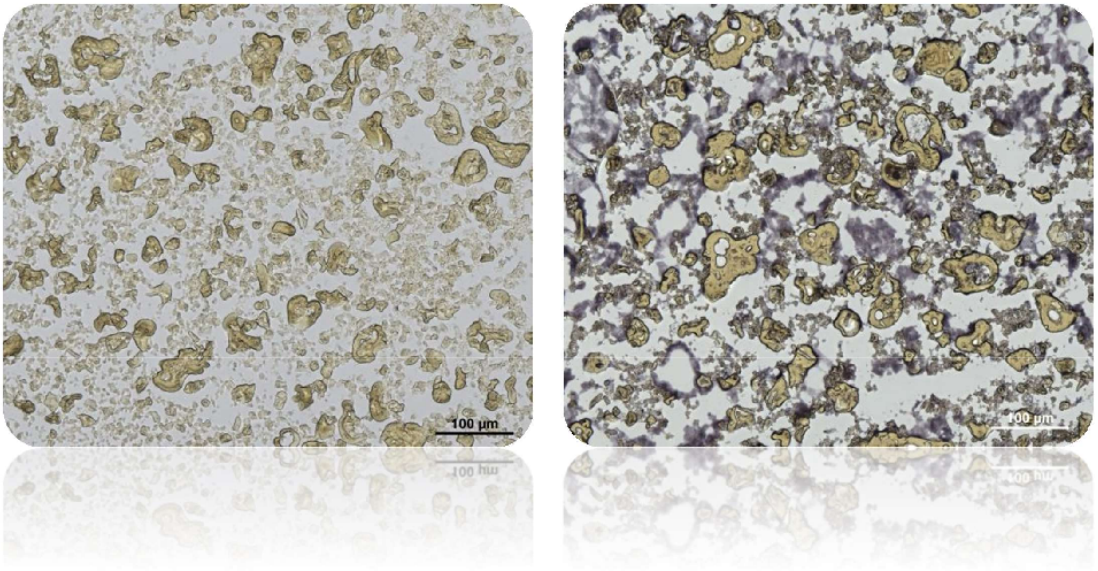

Pea protein-systems for plant based protein products

The effect of insoluble and soluble lentil fractions on rheology, microstructure and gelation properties of heat induced pea protein gels

Master's thesis in Biotechnology

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Cover: Light micrographs of heat induced pea protein gels, with (right) and without (left) the addition of insoluble lentil fraction, stained with iodine.

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Abstract

Mitigating climate change is one of today's generations largest challenges and the devastating effects that might follow can already start to be seen. As one of the largest contributors to climate change as well as the largest contributor to eutrophication, the food industry plays a key role in combating these issues. One way to reduce the environmental impact of our food is to reduce the consumption of animal derived foods and replace it with plant based foods, a shift in diet often referred to as the protein shift. However, for people to accept this dietary change, new products with similar nutritional value together with an equal or better consumer acceptance needs to be developed.

Peas (*Pisum sativum*) has recently gained an increasing interest due to its high protein content and overall high nutritional value. But in order for better utilisation of peas and pea protein, further research is needed. Heat induced protein gelation is a common process in the food industry. Understanding of this process together with characterisation of functional properties of relevant proteins can help in product development and optimisation of new and existing products. Research on heat induced pea protein gelation has so far been limited, especially regarding the effect of adding other food ingredients. In this context, a basic understanding of pea protein gelation and the effect of adding green lentil (*Lens culinaris*) fractions rich in soluble fibre (*soluble-fraction*) or insoluble fibre and starch (*insoluble-fraction*) prior gelation can be helpful. By analysing rheology, texture, gelation and microstructure, a good overview of the behaviour of these systems can be obtained and help in the developing of new plant bases foods.

The addition of insoluble- and soluble-fractions extracted from lentils was found to affect the texture, rheology and microstructure of heat induced pea protein gels. Addition of insoluble-fraction resulted in an increase of the fracture stress, Young´s modulus and storage modulus and many of the effects seemed related to the presence of starch. The addition of soluble fibre-fraction decreased the fracture stress and Young´s modulus of the gels but increased the storage modulus. Both fractions affected the microstructure of the gels. These results can help in development of new plant based foods in which protein gelation is critical for obtaining a desired texture.

Keywords: pea protein, gelation, dynamic rheology, starch, fibre, uni-axial compression, light microscopy

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Mathias Johansson, Gothenburg, September 2019

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Introduction

1.1 Background

In order to avoid many of the adverse effects due to climate change, a radical change in lifestyle is likely to be needed [1]. A significant part of the world´s total greenhouse gas emissions can today be allocated to the food production [2]. Multiple studies have shown the benefits, with respect to environmental impact, of a plant-based diet [2]. Environmental sustainability together with an increasing awareness of the ethical concerns with exploiting animals for food production is encouraging people to look for alternatives to animal derived foods. In this context, the demand of plant based foods is increasing every day and a protein shift from animal derived proteins to plant based alternatives seems to be inevitable.

To satisfy the increasing group of people consuming mostly plant based foods, new products need to be developed with the same or similar protein content without compromising on taste and texture. Part of this includes understanding of interactions between different components, such as proteins, within these processed foods, and the effects of different processing techniques and parameters. One function of proteins in foods is their property to form gels which plays an important role for the texture of many foods. However, the functionality of plant based proteins often differs from that of animal derived proteins. A better understanding of gel formation, and other processing techniques, of plant proteins could lead to further improvement and development of new vegan foods. Possible applications of plant protein gels may include gelled protein dessert and vegan cottage cheese-like products.

One particular functional ingredient which is increasingly being used in different types of newly developed foods is pea protein. In recent years the interest in pea proteins, for use in foods, have increased drastically in Sweden. It is used in a wide variety of products such as extruded meat analogues, ice cream and protein shakes. It could serve as an alternative for people allergic to soy or wheat which are commonly used as protein sources in plant based foods. Pea protein also has a high nutritional value, availability and low cost [3].

However, research done so far on pea protein and its function in and contribution to gels and other food matrices is limited. In order for a better understanding of the possibilities and limitation of using pea proteins in foods, further studies are needed. So far, most studies on pea protein gels have been investigating microstructure and

rheological properties related to pH, salt and protein concentration [4, 5, 6]. More studies on the interaction with other food components, such as fibres and fat are still to come. This thesis will focus on the effect of starch- and fibre-rich lentil fractions on the gelation of pea proteins.

There are multiple ways in which the project and the overall investment in research and development of plant based foods can be beneficial. An increased consumption of plant based protein instead of animal derived protein can improve health, reduce environmental impact and avoid some of the ethical issues with regards to livestock [2, 7]. Increased fibre intake have also been correlated to improved health [8].

However, a completely vegan diet is not always healthy for everyone. Certain factors such as metabolic diseases, mental illness, high age and low uptake of micronutrients can make it difficult to obtain a healthy diet by eating fully plant based. Other reasons making it difficult include economic reasons as well as farming possibilities in certain regions of the world. This together with the fact that a vegan diet lacks sufficient vitamin B_{12} (needs to be obtained via fortification or supplements), can be low on certain amino acids and micronutrients and might contain higher levels of anti-nutrients leaves some possible aspects for consideration before advocating a fully plant based diet.

This project aims to investigate how fibre- and starch-rich fractions extracted from green lentils affect the rheology, gelation and textural properties of pea protein gels at three different pH (3.0, 3.6 and 4.2). Knowledge on the behaviour of pea proteins in these systems can be helpful in the development of new plant-based foods. Different protein/lentil-fraction ratios will be analysed with respect to gelation and rheological properties and the impact of the lentil-fractions will be assessed using oscillatory rheology and compression tests. Investigation of the microstructure will also be performed, using light microscopy (LM), and correlated to the pH and addition of lentil-fraction.

2

Theory

Food science can often be complicated and multidisciplinary due to the complex matrices of many foods as well as the large differences between different types of foods. The scale on which the foods are investigated is also important depending on what knowledge is sought for. When studying gels, rheology is of great importance. However, rheology on its own does not explain what happens on a molecular or microstructural scale. In order to get a better understanding of the underlying cause of the changes in rheological properties it usually needs to be combined with other techniques, such as microscopy.

2.1 Pea protein, lentil fibre and lentil starch

Starch and proteins are both very important in foods, not only for nutrition and energy, but also when it comes to texture and processing. Their properties can be utilised in multiple ways resulting in a wide variety of products and textures. Proteins can be extruded to give a meat-like texture in plant based meat substitutes or heated in solution to form gels or puddings for dessert. In both examples, proteins are utilised to obtain a desired texture but resulting in products that differ greatly.

2.1.1 Functional properties of proteins

The functional properties of proteins can vary significantly, sometimes resulting in difficulties replacing one protein source with another without largely affecting the end result. This can lead to problems when trying to produce plant based product with similar properties as already existing ones based on animal derived ingredients. Hence, characterisation of plant based proteins is important in order to simplify this transition from animal to plant based proteins.

Functional properties of proteins can be seen as the physical and chemical properties of proteins affecting their behaviour in food systems during processing, storage, cooking and consumption [9]. The functional properties of proteins can be divided in some general classes including hydration (e.g. solubility, dispersibility, water absorption, swelling and thickening), organoleptic (e.g. color, flavor, odor, texture and mouth-feel) and rheological (e.g. aggregation, stickiness, gelation, texturizability, extrudability and elasticity) properties [9].

2.1.2 Pea protein

Globulins are the main proteins in pea seeds and make up about 70 % of the total protein content [3, 10]. The globulins and storage proteins legumin (11S) and vicilin (7S) are the two main protein groups in pea seeds [11]. Legumins are hexameric proteins with monomers consisting of one acidic (40 kDa) and one basic (20 kDa) subunit covalently linked by a disulfide bond [3, 11]. The monomers are non-covalently linked and the whole hexameric protein has a molecular mass of 300-400 kDa [3]. Vicilin proteins are trimers consisting of monomers (47-50 kDa) built up from three subunits [3]. Vicilin is held together by hydrophobic bonds and the whole protein has a molecular mass of 150-170kDa [3].

The ratio of legumin to vicilin have been found to vary significantly. Previous studies have reported ratios in the range of 0.5-6.2 [12, 13]. The ratio can vary due to many factors such as agronomic practice, harvest time and variety [14]. The ratio of legumin to vicilin is likely to affect the gelation of pea protein gels. The proportion of vicilin have been linked to the hardness of pea protein gels with increased hardness with increased proportion of vicilin [15].

Two important characteristics of proteins are the isoelectric point (IEP) and denaturation temperature. The isoelectric point and lowest solubility of pea protein is usually found in the range from pH 4-6, with some variation depending on extraction method, variety, etc. [16, 17]. There is also a difference in the IEP of vicilin and legumin protein fractions with IEP at 5.5 and 4.8 respectively [11]. The denaturation temperature of pea protein have been reported as 85.1°C from differential scanning calorimetry measurements using a heating rate (10 °C/min) [18]. Slightly higher denaturation temperatures, 90.5-93.3°C, have also been reported using a lower heating rate (0.5-4 °C/min) [19].

The functional properties of pea protein is easiest described in relation to other proteins. For example, pea protein has been compared to kidney beans in terms of solubility, emulsifying and foaming properties. It was found to have higher water and fat holding capacities whereas kidney bean protein made stronger gels [20]. Comparing to the widely used soy protein, pea protein have been found to have a lower water holding and oil holding capacity [16].

Solubility, foaming and emulsifying properties of different varieties of pea, adzuki bean and soy protein isolates have also been studied. Overall, the different soy varieties showed higher solubility irrespective of pH, native soy formed more stable foams at all pH and the legume with the best emulsifying properties varied depending on pH [21].

Regarding gelation, pea protein requires a higher concentration to be able to form a gel than soy protein as well as higher or equal concentration as chickpea protein [16]. When prepared under the same condition as soy, the gels from pea protein becomes weaker and less elastic [16, 18].

However, all these properties vary depending on multiple variables such as variety, pH and extraction method. The most suitable protein isolate for a specific product therefore depends on the processing technique and the desired properties of the food.

2.1.3 Starch and fibre in lentils

Lentils contain around 42g starch per 100g (dry weight) with about four of these grams being resistant starch [22]. Starch consist of two molecules, amylose and amylopectin usually present as granules. When cooking starch the granules will swell, amylose leak out and the starch will gelatinize. The gelatinization of lentil starch has been reported to occur around 70°C with the onset just above 60°C and end of gelatinization just below 80°C [23, 24].

Multiple studies have reported the amount of soluble and insoluble fibre in raw lentils. The content of soluble fibre is usually found around 1-3 % (dry weight) and insoluble between 19-22 % (dry weight) [25, 26]. Cellulose, xylans and arabinans have been found to be the most present insoluble fibrs in lentils and are primarily found in the husk [22]. The soluble fibres consist of mainly arabinose and galacturonic acid rich pectins together with some hemicelluloses and some galacturonans [22]. Where the most abundant one, pectin, is a commonly used gelling agent in foods.

2.2 Gels and gelation

Gels can form in multiple ways. The most common processes in foods include heat-induced gelation of proteins or polysaccharides such as starch. Starch forms gels by first absorbing water leading to swelling of the starch granules during heating and water is gradually absorbed in an irreversible manner. The amylose crystalinity will then start to break and primarily amylose leaks out into the solution forming a paste that eventually forms a gel during cooling [27]. Globular proteins can often form gels via denaturation followed by interaction between recently exposed interaction sites leading to aggregation and later agglomeration. The denaturation can be induced by different means such as heat, acid or enzymes. Heat induced protein gelation is explained further in section 2.2.1.

The definition of a gel can be somewhat ambiguous. After a thorough review of existing definitions and descriptions *Almdal et al. (1993)* [28] arrived at the following criteria that should be fulfilled for systems to be defined as gels:

> *(a) A gel is a soft, solid or solid-like material of two or more components one of which is a liquid, present in substantial quantity.*
>
> *(b) Solid-like gels are characerized by the absence of an equilibrium modulus, by a storage modulus, $G'(\omega)$, which exhibits a pronounced plateau extending to times at least of the order of seconds, and by a loss modulus, $G''(\omega)$, which is considerably smaller than the storage modulus in*

the plateau region.

The terms storage modulus (G′) and loss modulus (G″) are explained in more detail in section 2.3.1. However, in a simplified way G′ can be described to represents the elastic portion or the energy stored in the elastic structure of the sample. In the same way G″ represents the viscous part or the amount of energy the sample loses as heat. A storage modulus significantly larger than the loss modulus is true if the material is solid-like and resilient and a storage modulus plateau extending over seconds is true for viscoelastic solids [28]. The extending of the storage modulus plateau can be seen as that the gel should stay "solid-like" for at least seconds when a deformation (not too large) is applied to it, i.e. the structure should not collapse.

2.2.1 Heat induced protein gelation

One way by which protein gels can be obtained is, as previously mentioned, by heating of protein solutions. If the protein concentration is high enough and the functionality of the protein allows, gels can form when protein solutions are heated above the protein denaturation temperature. The heating must be sufficient for the proteins to unfold/denature, at least partially, to form new bonds with surrounding proteins.

The heat induced gelation process of globular proteins can often be summarised in the three following general steps [29]. First, the proteins are denatured due to the increased temperature and hydrophobic residues and other interaction sites are exposed. Secondly, the proteins starts to aggregate due to interactions between the recently exposed interaction sites, and lastly the aggregates start to agglomerate to form a network. During the cooling of the gel the network is further strengthened due to formation of hydrogen bonds and other short-range interactions [30].

2.2.1.1 Heat induced pea protein gelation

Previous research on heat induced pea protein gelation have investigated the effect of pH, protein concentration, salt concentration, heating and cooling rate and protein extraction method [4, 6, 19, 31]. A higher protein concentration, in the range of 100-150 mg/mL, results in stronger and stiffer gels [4]. An increase in pH, between pH 3-4.2, results in decreased fracture stress and weaker gels and a structural change from finer to coarser protein networks was found at around pH 3.7, i.e. when approaching the IEP [4]. This structural change, from a coarse stranded to a fine stranded network is believed to be one of the main reason for the change in rheological properties of the gels.

Munialo et al. (2015) could also relate the size of the beads (small spherical aggregates) and the strings (thread-like strands/filaments connecting the beads) making up the protein network to the rheological properties of the gels [4]. The same study also showed that structural changes of pea protein networks are largely determined

by the interplay between the aggregation of the pea-proteins and the agglomeration of the aggregates [4]. An increase in aggregation speed compared to agglomeration speed gives a more coarse stranded network which largely effects the rheological properties of the gels [4]. And similarly, a slower rate of aggregation relative to denaturation gives a more fine stranded gel network [32]. Two of the most important factors affecting weather the resulting protein network will be coarse- or fine-stranded are the pH and ionic strenght. Fine stranded gels are in general formed at pH away from the isoelectric point or at low ionic strength [33].

Molecular forces involved in heat induced gels from salt-extracted pea protein isolates have been investigated by *Sun et al. (2012)* [34]. They showed results indicating that non-covalent bonds have a significant role in gel formation. During gel formation, electrostatic and hydrophobic interactions seemed to be the main contributors to the initial structure development, and hydrogen bonds the main contributor to the stiffening of the gel during cooling. Disulphide bonds were also investigated and found to have a minor effect on the gel characteristics during certain processing conditions but not to be necessary for the gel formation.

2.2.2 Combined pea protein and polysaccharide gels

Very few studies have so far been conducted focusing on the effect of adding starch or fibre to pea protein gels. Since food matrices are often quite complex and containing not only protein, information and understanding of interactions with and effects of addition of other common food constituents is of great importance. One of the few studies available have investigated the rheological properties of mixed gels from pea protein and κ-karrageenan where it was seen that κ-karrageenan can increase the strength of pea protein gels [35].

No studies were found on mixed pea protein and starch gels. Studies have however investigated other protein and starch gels. Cassava starch at relatively low concentrations have been found to increase the storage modulus of heat induced whey protein gels [36]. Adding a too high concentration of corn starch has been found to have a negative effect on the storage modulus of soy protein gels due to starch disturbing the protein and creating holes in the continuous protein network [37].

2.3 Rheology

Rheology is the study of flow and deformation of matter and is important not only in food science but also many other areas such as polymer science. This section will mainly focus on rheology relevant to viscoelastic foods and gels in particular.

2.3.1 Stress and strain

Rheological properties are often derived from stress and strain measurements. Stress is the force applied to the sample and strain a description of the deformation. When

a tensile or compression force is applied to a sample, the stress and strain are often referred to as σ and ε respectively and can be calculated according to equation 2.1 and 2.2,

$$\sigma = \frac{F}{A} \qquad (2.1) \qquad\qquad \varepsilon = \frac{\Delta L}{L} \qquad (2.2)$$

where F (N) is the tensile or compression force, A (m^2) the area to which the force is applied, ΔL (m) the deformation and L (m) the length of the specimen.

However, for ductile samples where the surface area will change throughout the measurement, the stress and strain needs to be converted into true stress and true strain while assuming constant volume. True stress and true strain are defined as in equation 2.3 and 2.4, where A_i (m^2) is the initial area, L_i (m) the initial length of the specimen, F is again the applied force and ΔL is the difference between the current and initial length of the specimen [5].

$$\sigma_T = \frac{F}{A_i} \times \frac{L_i - \Delta L}{L_i} \qquad (2.3) \qquad\qquad \varepsilon_T = ln\left(\frac{L_i}{L_i - \Delta L}\right) \qquad (2.4)$$

When a compression force is applied to a gel it will first start to deform without any large scale fracturing. During this part the stress will increase proportionally to the strain. When the deformation and force is increased the gel will eventually start to break and crack. The force at which the gel breaks is called the fracture stress, and the corresponding strain is referred to as the fracture strain. Fracture stress can be seen as a measurement of the gel strength and fracture strain a measurement of the brittleness of the gel. A stress versus strain curve for a weak heat induced pea protein gel can be seen in Figure 2.1 together with a representation of the true fracture stress, true fracture strain and the Young´s modulus. The true fracture stress and true fracture strain will from hereon be designated simply as fracture stress and fracture strain respectively.

A modulus is the ratio of stress to strain, i.e. the ratio of force and deformation. Young´s modulus is the ratio between stress and strain, in the linear region, during uniaxial compression, see Figure 2.1 and is a measurement of the elasticity or stiffness of a material [38]. The larger the Young´s modulus, the smaller deformation on a given stress. Accordingly a material with a small Young´s modulus would elastically deform more easily. [38].

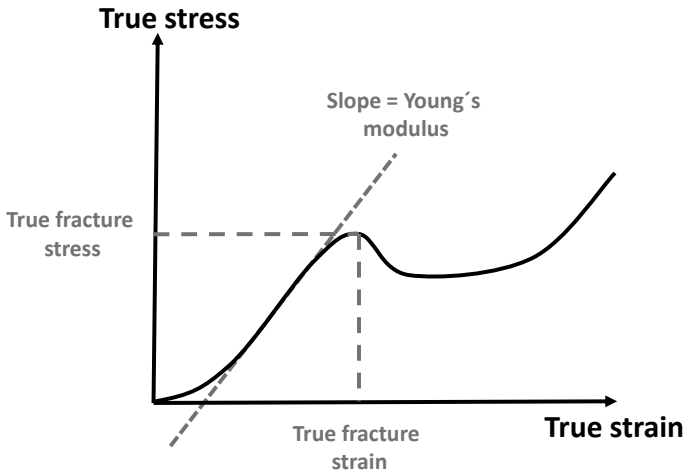


Figure 2.1: Typical stress versus strain curve from a compression test on a pea protein gel, including definition of fracture strain, fracture stress and Young´s modulus.

When referring to shear stress and shear strain the symbols τ and γ are sometimes used instead. In a simple system with two parallel plates and the sample in between the shear stress (τ) and shear strain (γ) can be defined as in equation 2.5 and 2.6 together with the variables defined as in Figure 2.2.

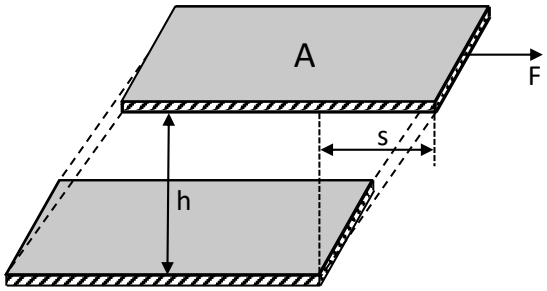


Figure 2.2: Figure visualising variables used to define shear stress and shear strain. A is the surface area, F is the shear force and s and h are distances describing the relation between the two surfaces.

$$\tau = \frac{F}{A} \qquad (2.5) \qquad\qquad \gamma = \frac{s}{h} \qquad (2.6)$$

Where F (N) is the shear force, A (m^2) the area to which the force is applied, s (m) the deformation and h (m) the distance between the two plates.

2.3.1.1 Uniaxial compression

As mentioned, stress and strain can be important measures when analysing the texture properties of a material. One way to obtain these measures is by using a texture analyser. Depending on the chosen setup, different tests can be performed and different properties analysed. Such tests can include tensile, compression, bend and peel tests and give valuable information about your material or product.

Uniaxial measurements are measurements performed along one axis. Applying a compression force along one axis and recording the stress-strain relationship can, as mentioned earlier, be used to estimate the strength, brittleness and elasticity of a gel.

2.3.1.2 Dynamic rheology

Instead of using tensile or compression forces to deform a material, shear forces can be used. This allows to measure the viscosity of solutions and can be performed using a system with a cup and an inner cylinder similar to what is seen in Figure 2.3. By rotating the outer cup containing the liquid, the inner cylinder submerged in the sample will follow. Depending on the viscosity of the solution, the inner cylinder will spin with different speeds and be exposed to different forces. The higher viscosity the faster the inner cylinder will spin.

However, if we want to measure materials that are not always behaving as liquids, viscosity is not enough to characterise them and spinning the cup will destroy the structure of the material we want to measure. Consider for example an egg white that is being heated. First it will behave like a liquid, but as the temperature is increased the egg white will eventually coagulate and become solid. To avoid the destruction of the sample, the cup can instead be oscillated in a sinusoidal pattern. If the oscillations are small enough, the sample will stay intact and measurements can continue also after coagulation of the egg white

Oscillating measurements will give not only the viscosity, but also the complex shear modulus G* which can be divided into the storage modulus (G′) and loss modulus (G″) according to equation 2.7. The magnitude of the complex shear modulus, G* is given by the shear stress amplitude, τ_0 (Pa), divided by the shear strain amplitude, γ_0 (dimensionless) and together with the phase angle, δ, describes the entire viscoelastic behaviour of the sample [39].

$$G^* = \frac{\tau_0}{\gamma_0} \times e^{i\delta} = G' + iG'' \tag{2.7}$$

The storage and loss modulus can be calculated from the complex shear modulus magnitude and the phase shift (δ) between the applied strain and the measured stress according to equation 2.8 and 2.9 [39].

$$G' = \frac{\tau_0}{\gamma_0} \cos \delta \tag{2.8}$$

$$G'' = \frac{\tau_0}{\gamma_0} \sin \delta \tag{2.9}$$

For further details on the derivation of these equations the reader is referred to *Ferry (1980)* [39].

Storage modulus (G′) and loss modulus (G″) are commonly used to describe viscoelastic materials, i.e. materials that cannot solely be described as neither solids nor liquids. The storage modulus can be seen as the elastic portion and the energy stored within the structure of the sample, and the loss modulus as the viscous portion and the energy dissipated as heat.

A higher storage modulus means a higher ability to store energy within the material and requires some sort of internal structure or network [40]. The energy can be stored for example by extending and stretching internal structures without destroying them. When the applied force is released, the stored energy will help reforming the structure towards its original shape [40]. A material with a G′ larger than its G″ is referred to as a viscoelastic solid and when the opposite is true, the material is referred to as a viscoelastic liquid. [41]

A simplified version of a dynamic rheometer can be seen in figure 2.3 together with a visualisation of the phase shift between the applied sinusoidal shear strain, γ and resulting shear stress, τ. The shear strain is applied via the rotating outer cup and the torque measured from the inner bob is converted to shear stress via a conversion factor specific for the used setup. From these two values, together with the phase shift angle δ, the complex shear modulus can be obtained.

Tan(δ) is referred to as the loss or damping factor and is the ratio of G″ to G′. For an ideally elastic material δ equals to zero giving a G″ also equal to zero. For an ideally viscous material, δ equals to 90°and thus G′ equals to zero. For heat induced protein gels, the gelling temperature is often referred to as the temperature at which the storage modulus exceeds the loss modulus. [41]

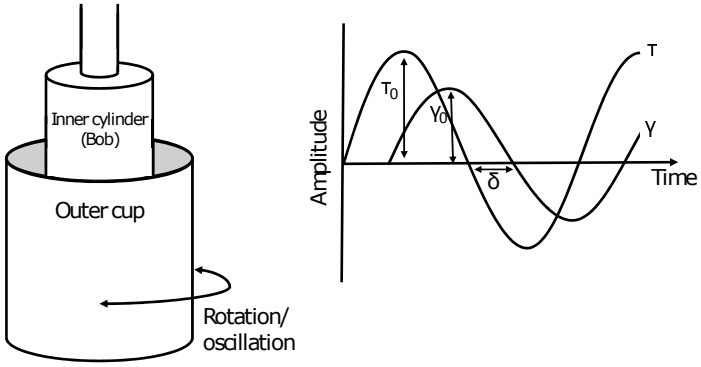


Figure 2.3: A simplified figure of a rheometer together with a visualisation of the phase shift between the applied shear strain (γ) and measured stress (τ).

A bob in a cup is not the only setup that can be used for dynamic rheology measurements. Two parallel plates or a plate and a cone are two other possible setups. Different setups are used for different materials and different purposes but are all based on the same principle. Ideally the same result should be obtain independent of geometry. However experimental limits exist as well as other factors such as differences in strain dependence, making certain geometries preferable to others in specific cases [42].

2.3.2 Rheology in foods

Rheology is used in food science and food industry to quantitatively evaluate and compare different foods in terms of viscosity and texture. It is used when designing equipment and has relevance to the customer´s experience of the food [38]. For example, rheology is of great importance for the mouth-feel of many foods as well as for designing food for people with certain swallow disorders [43].

Rheological properties can be correlated to the texture and perception of foods. For example, recoverable energy has been shown, together with microstructure, to correlate to the crumbly perception of whey protein/polysaccharide gels [44]. Other studies have shown connections between the perception of firmness and the fracture stress and fracture strain of different food gels [45]. G′and G″correlate to perceived thickness, glueyness and sweetness in pectin gels with added sugar [46].

Knowing how to control these physical parameters and correlating them to perception can help to design a food product with desired properties. It should however be

noted that the perception of food is a complicated science and is often a result from the combination of many physical properties of the food. Hence, finding correlations between rheological properties and perception are often a difficult task.

2.4 Microscopy

Microscopy is widely used in food science. It is a useful tool to understand the structure and properties of a food and is often used in combination with other techniques. Microscopy can help in understanding differences in structure between two processing techniques or help understanding the mechanism and interaction between compounds affecting the texture of the food.

Microscopy can in some cases also be used to correlate the structure to sensory properties or other attributes. For example, the microstructure of whey protein gels has been shown to correlate to sensory properties such as how gritty or creamy the gels are perceived [47]. Noted should however be that these types of correlation are, just as for rheology, hard to make and the conclusions that can be made are limited. The sensory and perception of food is a very complex issue and care should be taken not to draw the conclusions to far.

The microstructure of pea protein gels have also been used to explain changes in large scale deformation rheological parameters such as fracture properties and recoverable energy. *Munialo et al. (2014)* found a correlation between the transition from a finer to a coarser protein network (on a 100 nm scale) and rheological data [5]. Hence, microscopy and an observed change in microstructure could give valuable knowledge of the structure of a food relevant for its functional properties.

2.4.1 Light microscopy

Light microscopy (LM) utilises visible light and magnifying lenses in order to visualise small objects with a resolution higher than for the eye. The resolution of light microscopy is in theory limited by the wavelength of the light, and in practice also the objective quality [48]. One drawback with light microscopy is the requirement of a thin sample which sometimes introduce problems due to preparation altering the structure of the sample.

Iodine, usually in the form of Lugol´s solution, is commonly used for staining of starch when using light microscopy. The proposed action involves intercalation of iodine ions inside the starch, or more specifically the amylose helices, giving rise to the change in colour [49]. The Iodine will stain amylose in blue and amylopectin in weak brown or violet [50]. Iodine also stains proteins in yellow [51].

2.4.2 Confocal laser scanning microscopy

Confocal laser scanning microscopy (CLSM) uses focused laser with specific wavelengths rather than the full spectra of visible light to illuminate the sample. The scanning laser illuminates part of the surface of the sample and the instrument is constructed in such a way that only reflected fluorescent light from a defined focal plane is able to pass back to the detector. This creates an image of a optical slice of the sample with higher resolution than for conventional light microscopy. By moving the sample up and down relative to the focused laser, consecutive optical sections and three-dimensional information can be obtained [52].

CLSM is based on excitation and fluorescence of fluorophores. If the sample does not fluorescence itself, or one wants to distinguish between different compounds, staining has to be used. Rhodamine B is a fluorophore commonly used to visualise protein using CLSM [53].

Since CLSM does not depend on transmitting light through the specimen, bulk samples can be used instead of thin sections. This removes some of the possible issue with sample preparation damaging structural elements during sectioning.

2.5 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a technique used to measure the amount of heat needed to change the temperature of a sample. It is a common technique in food research to analyse transition points such as the transition of a protein from one conformational state to another [54]. Other applications include studying of starch gelatinization, melting point of lipids and glass transition of different foods [54]. DSC is not limited to proteins nor food science, it can also be used to study other food components and processes [54].

A typical graph of DSC data for a dissolved protein can be seen in Figure 2.4, where heat flow (mW) is plotted versus temperature (°C). The transition temperature (T_m) is defined as the peak heat flow and a higher T_m indicates higher thermostability. In the case of protein measured around its denaturation temperature the transition midpoint represents the denaturation temperature (T_d). In a complex food system, this temperature can be affected by multiple factors such as water content, pH, sugars and salt concentration [54].

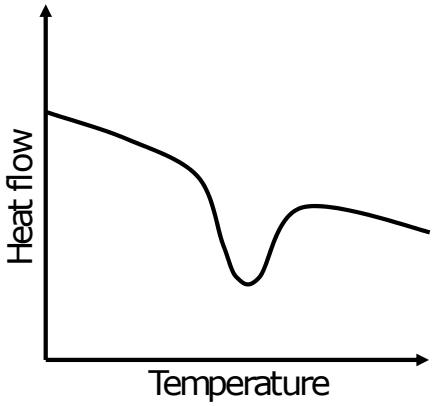


Figure 2.4: A typical DSC graph for a protein in solution around its denaturation temperature.

2.6 Moisture loss

The ability of foods to hold water is often an important feature. For gels, a low ability to hold water can result in shrinking of the gel and changed texture resulting in a product of lower quality. Moisture loss and other water holding properties can be estimated in multiple ways. One common way is by using a filter or membrane together with centrifugal force to force some of the water out of the sample. Sometimes a consecutive drying step is also included. The better the food matrix can hold the water, the less water will be released. In this report, the results will be reported as moisture loss according to equation 2.10.

$$ML = 1 - \frac{w_{final}}{w_{initial}} \tag{2.10}$$

Where ML is the moisture loss, $w_{initial}$ the weight of the sample before centrifuging and w_{final} the weight of the sample after centrifuging.

When considering moisture loss of gels it is of importance for the gels to maintain their structure during the centrifuging [55]. This is taken into account since the measurements should represent the ability of the gel structure to hold water rather than its components as such. Therefore, high-speed centrifuging is often avoided for determination of water binding properties of gels [55].

The forces involved in maintaining the water within the structure vary depending on the properties of the gel. In general, coarse stranded gels will lose water when the applied force exceeds the one of the capillary pressure. Whereas for fine-stranded gels the capillary pressure is to high and water release is mainly due to syneresis. [56]

3
Method

The main techniques used within the project included dynamic rheological measurements, gel strength measurements by compression tests, light microscopy and extraction of soluble fibre rich soluble-fraction and starch rich insoluble-fraction from dry lentils. Dynamic rheological measurements was performed measuring the storage modulus (G′) and loss modulus (G″) during gelling and uniaxial compression measurements to measure the Young´s modulus, fracture stress and fracture strain on prepared gels. Light microscopy was used to analyse the microstructure of the pea protein gels and verified by confocal laser scanning microscopy.

3.1 Material

The pea protein used was a commercial pea protein (NUTRALYS® S85F pea protein isolate) from *Pisum sativum* with a protein content of minimum 84 % (dry basis). Soluble fibre-rich and starch and insoluble fibre-rich lentil fractions (referred to as *soluble-fraction* respectively *insoluble-fraction* throughout this report) were extracted from green lentils (Gröna Linser, Saltå kvarn, 500g), *Lens culinaris*, purchased at a local supermarket. HCl and NaOH used for extraction was of food grade (FCC) and ethanol as well as HCl for pH adjustment of solutions of extra pure grade. Deionized water was used for the extraction and all solutions were prepared with MilliQ water.

3.2 Extraction

The insoluble-fraction was extracted from green lentils as follows. 100g of green lentils was mixed using a knife mill (RETSCH Knife Mill Grindomix GM200) for 3x20s at 7500 rpm giving a coarse flour. The pH of 350 mL deionized water was adjusted (SevenGo Duo pro combined with a InLab Expert Pro-ISM-IP67 pH electrode, Mettler Toledo) to 2.5 using 0.5M HCl before slowly adding 35g of the lentil flour while simultaneously keeping the pH at 2.5 using 0.5M HCl. When all the flour had been added, the pH was checked and adjusted after 5 and 10 minutes before stirred for an additional 60 minutes. The solution was then centrifuged (Heraeus Megafuge 16R centrifuge with a TX400 rotor, Thermo Scientific) at 3600 rpm (2434g) for 20 minutes and the supernatant (protein- and soluble-fraction) was separated from the pellet (insoluble-fraction). The pellet was put in a freezer (-80°C) until freeze drying.

The pH of the supernatant was adjusted to 4.3 (Isoelectric point green lentils [57]) using 1M NaOH for precipitation of the protein and centrifuged (3600 rpm (2434g), 20 min). The pellet (protein) was separated from the supernatant (soluble-fraction) and washed once by dissolving in deionized water (volume ratio of 1:10, pellet:water) for 30 minutes before centrifuging (3600 rpm (2434g), 20 min). The supernatant was discarded and the pellet frozen (-80°C) before freeze drying.

The soluble-fraction was extracted, based on a method used by *Brummer et al. (2015)* [22], from the supernatant obtained after the protein precipitation by mixing with 95 % ethanol at a volume ratio of 1:4 (supernatant:ethanol(95 %)) before centrifuging (3600 rpm (2434g), 20 min). The supernatant was discarded and the pellet frozen (-80°C) before freeze drying.

All the resulting extracts (protein, soluble-fraction and insoluble-fraction) were, after frozen at -80°C, freeze dried (Alpha 1-2 LDplus freeze dryer, Martin Christ). The freeze drying was run for 24 plus 22 hours at 34 mbar and -31°C with a careful grinding of the resulting dry cake and cooling (-80°C freezer) of the sample after the first 24 hours.

Some of the insoluble-fraction was also sieved (Vibratory Sieve Shaker ANALYSETTE 3, Fritsch) with a 125 µm sieve. This fraction will be referred to as *sieved insoluble-fraction* and was used for the dynamic rheology measurement as well as some microscopy. The non-sieved insoluble fraction was used mainly for the uni-axial compression tests and microscopy.

3.3 Preparation of pea protein gels

Gels and protein solutions were prepared using pea protein isolate (minimum 84% protein) for a final concentration of 150 mg PPI/mL water. This concentration was chosen based on some initial testing at which the available PPI was found to form self-standing gels at pH 3 and a concentration between 100 and 150 mg PPI/mL water after heating for 30 minutes at 95 °C. For the samples including insoluble-fraction or soluble-fraction, these fractions were added before the protein and dissolved for 15 minutes while stirring. Insoluble fraction was added at ratios of 0.1, 0.2 and 0.3 gram insoluble-fraction per gram PPI and soluble-fraction at ratios of 0.1 and 0.2 gram soluble-fraction per gram PPI (e.g. the *"0.1 soluble-fraction"*-samples contain 150 mg PPI plus 15 mg soluble-fraction per mL water). The protein was then added and dissolved under mixing for an additional 1.5h. Thereafter the pH of the protein solution was adjusted to three different pH (3, 3.6 and 4.2) before heated or analysed.

For the heat-induced gelation, volumes of about 2.8 mL solution was transferred to glass cylinders, diameter of 16 mm, with rubber stopper at the bottom as well as a rubber lid with a small hole on top, see Figure 3.1. The samples were then placed in a water bath at 95 °C for 30 minutes before taken out and left in room temperature overnight.

Figure 3.1: Glass cylinders with rubber stopper and rubber lid with a small hole used for preparation of pea protein gels.

3.4 Rheology

Methods used for rheology measurements were based on procedures used by *Munialo et al. (2015)* with some slight modifications [4].

3.4.1 Uniaxial compression

Compression tests were performed on gels (5-9 replicates) prepared and heat treated according to section 3.3. Young´s modulus, fracture stress and fracture strain were all obtained from the measurements. The measurements were performed using an advanced material testing system (INSTRON 5542) with a 500N static load cell and circular probe with a diameter of 30 mm. The compression speed was set to 0.1 mm/s and sandpaper with a grit size of 180 was used on both the surface of the probe and the bottom plate in contact with the sample to keep the gels in place during testing.

3.4.2 Dynamic rheological measurements

Dynamic rheological measurements were performed to measure the storage modulus (G') and loss modulus (G'') during *in situ* thermal gelation using a rotational rheometer (ARES-G2, TA Instruments). This was done using the same thermal processing scheme as in *Munialo et al. (2015)*, which is supposed to resemble the thermal processing experienced by the gels prepared according to section 3.3 [4]. Measurements were started by carefully adding about 20 mL solution, prepared according to section 3.3, into the cup at room temperature. Before the actual temperature ramp started, the sample was held at 20 °C for 1 min. The temperature ramp started with heating to 95°C at a constant rate of 5°C /min followed by a 30 minute holding time. Thereafter the temperature was decreased with a constant rate of 1 °C/min before held at 20 °C for 30 min.

The measurements were performed using an oscillating cup with a diameter of 30 mm and a fixed DIN bob with a 27.7 mm diameter. The gap was set to 5.854 mm and a maximum strain of 0.5 % and angular frequency of 6.28 rad/s (1 Hz) was used. No solvent trap or low density oil was used to prevent evaporation. Due to the relatively long holding time at 95°C (30 minutes), evaporation did affect the measurements.

During the entire thermal treatment, the storage modulus (G′) and loss modulus (G″) were measured. Testing frequency and strain was based on previous studies together with results from strain and frequency sweeps. The strain was chosen to be within the linear viscoelastic response region (LVER) based on amplitude sweeps on gels following the thermal treatment described earlier in this section. The LVER was defined as the region where the strain causes 5 % or less reduction compared to the initial storage modulus.

Some measurements were also performed using a parallel-plate system (results not included) with a circular 40 mm upper plate. The gap was set to 1mm, maximum strain to 0.5 % and the angular frequency to 6.28 rad/s (1 Hz). To minimise evaporation, a solvent trap with MilliQ water and a thin layer of paraffin oil covering the air exposed edges of the sample was used. However, due to large variations in final G´-values and a retarded gelation during the cooling phase for some replicates, this setup was discarded in favour for the concentric cylinder setup.

3.5 Microscopy

Light microscopy was used to study the microstructure of the pea protein gels and the effect of starch and fibre. CLSM was used to confirm that cryo-sectioning did not heavily alter the microstructure of the gels.

3.5.1 Light microscopy

Samples for light microscopy were prepared by fast freezing with liquid nitrogen and cutting using a sectioning cryostat (LEICA CM1900). The gels prepared according to section 3.3 were frozen by dipping quickly multiple times in liquid nitrogen. The samples were then mounted and placed in the cryostat and left for a minimum 30 minutes to equilibriate with the cryostat temperature set to -14°C. Sections with a thickness of 9 µm were cut and placed on microscope slides for staining and microscopical analysis.

Iodine in the form of Lugol´s solution was used to stain the protein (yellow) and the starch (purple/dark blue). Pictures were taken with a DFK 33UX264 5 megapixel camera connected to a Nikon Microphot-FXA light microscope using the software NIS-Elements D 5.10.

3.5.2 Confocal laser scanning microscopy (CLSM)

CLSM was used mainly to confirm that gels were not affected during sample preparation for LM (freezing and cryo-sectioning). This could be done by observing gels directly under the microscope without any sample preparation other than staining, cutting a suitable piece using a razor blade and transferring it to a microscopic slide.

Gels prepared as in section 3.3 were stained using a 20 µL/mL aqueous solution of Rhodamine B (0.2% w/w) before cut as flat as possible and placed on a microscopic slide for analysis. A few samples were also prepared by adding 20 L/mL aqueous solution of Rhodamine B (0.2% w/w) before heating and gelation of the protein solutions.

Micrographs were obtained using a Leica TCS SP2 confocal laser scanning microscope configured with an upright microscope using an HeNe laser for excitation at 543 nm. Emission was recorded between 550 and 620 nm.

3.6 Differential scanning calorimetry

Thermal properties of pea protein solutions with and without addition of insoluble fraction was examined using differential scanning calorimetry similarly to *Shand et al. (2007)* with some slight modifications. Measurements were performed using a differential scanning calorimeter (DSC 1 Star system, Mettler Toledo). Protein solutions (1, 3 and 15 %, w/v) were prepared as previously described in section 3.3, with (pH3) or without (approximately pH7.5) the pH adjustment. A droplet of sample (5-12 µg) was placed in the centre of a 40 µL crucible which was then hermetically sealed. Measurements were run from 20 to 120 °C with a heating rate of either 3 or 10 K/min. A sealed empty crucible was used as reference.

3.7 Moisture loss

The moisture loss was defined as one minus the ratio between the weight of the sample after centrifuging to the weight of the sample before centrifuging, see equation 2.10. Gels were prepared according to section 3.3 with the exception of a smaller sample volume (ca 1.2 mL). The gels were carefully placed on a filter paper before placed on a nylon net (mesh width 715 µm) and centrifuged (500 rpm (42g), 10 min using a Universal 320 centrifuge, Hettich Zentrifugen). Analysis was performed on 2-6 replicates. Hence the reliability of the results is quite low and the results are only indicative of what further studies might find.

Samples were place standing on the filter paper in the same direction as they stood in the glass cylinders when made, i.e with the bottom face down on the filter paper. This due to partial drying giving a harder structure on the surface which to some extent could prevent the "free" water from escaping the gel downwards.

3.8 Statistical analysis

The statistical software XLSTAT (add-on for Excel) was used for statistical analysis of collected data [58]. ANOVA and pairwise comparison (Tukey (HSD)) was used for analysing the data.

4
Results & discussion

It should be noted that the experiments are performed on non-refined materials. This can complicate the understanding and interpretation of the results, but also be beneficial if a desired change in functionality can be obtained without the water and energy intense purification often needed to produce a refined material. Notice should also be taken to the fact that the insoluble-fraction and soluble-fraction have not been fully characterised. Their assumed contents are instead deduced from analysing them using light microscopy as well as comparing with contents reported in extracts obtained using similar extraction methods.

4.1 Extraction

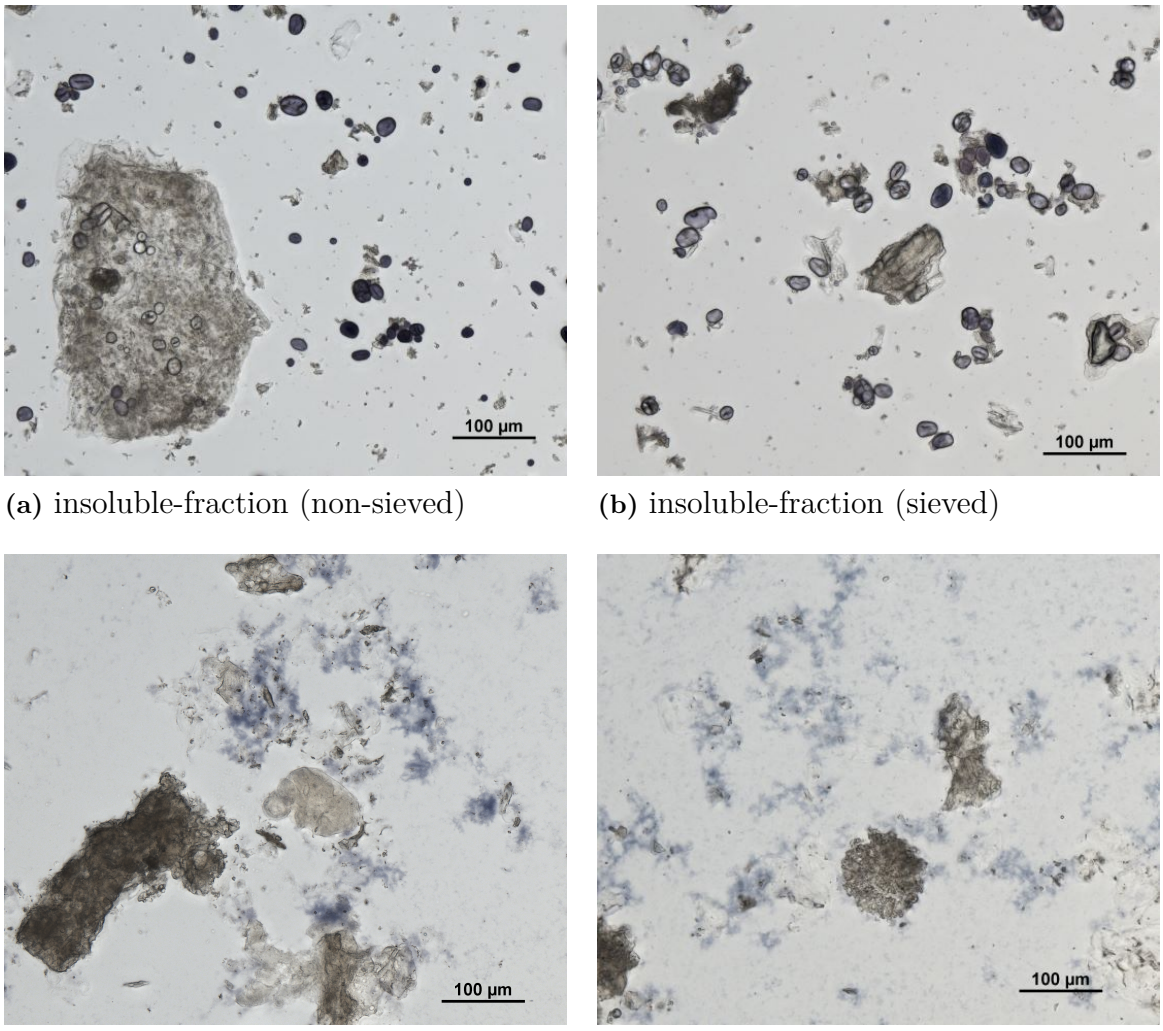
The extracted soluble- and insoluble-fractions were studied using light microscopy to get a better understanding of their contents. Both the sieved and non-sieved insoluble-fraction was analysed as well as the soluble-fraction. The effect of the heat treatment (water bath at 95°C, 30 min) was also investigated.

The light micrographs in Figure 4.1 of solutions containing only insoluble-fraction clearly shows the presence of starch granules (light purple/blue). There is also some unstained material present, most likely consisting of mainly insoluble fibre, husk pieces and cell wall residues. In addition to that, the non-sieved insoluble-fraction was found to contain some even larger pieces (>125 µm) originating from the lentil husk as well as some groups of cells still attached to each other.

Comparing the micrographs for the cooked (figure 4.1 (a,c)) and non-cooked (figure 4.1 (b,d)) insoluble-fraction, the clear swelling and degradation of the starch granules resulting in release of amylose and amylopectin can be observed. Another observation is that the many of the larger pieces of non-stained material seems to retain their structure after cooking. It can also be noted that the gelatinised starch does not form any clear structures or aggregates resembling the ones found when included in the protein gels (see Figure 4.13).

However, for one of the replicates of the cooked insoluble-fraction, some of the starch was still present as what looks like swelled and deformed starch granules, see Figure A.3 in Appendix A.2. When a new replicate from the same sample was prepared, a structure similar to Figure 4.1 (b) was observed. This might be due to that the

cooked and swelled granules are sensitive to something during the preparation for microscopy. Possibly mechanical stress and breakage during mixing with the staining solution. High shear rate during cooking have previously been shown to break the residual granule structures sometimes referred to as "granule ghosts" that can be present after gelatinization [59]. The shape of these swelled granules does to some extent resemble the starch seen in the protein gels in section 4.4.1.



(a) insoluble-fraction (non-sieved)

(b) insoluble-fraction (sieved)

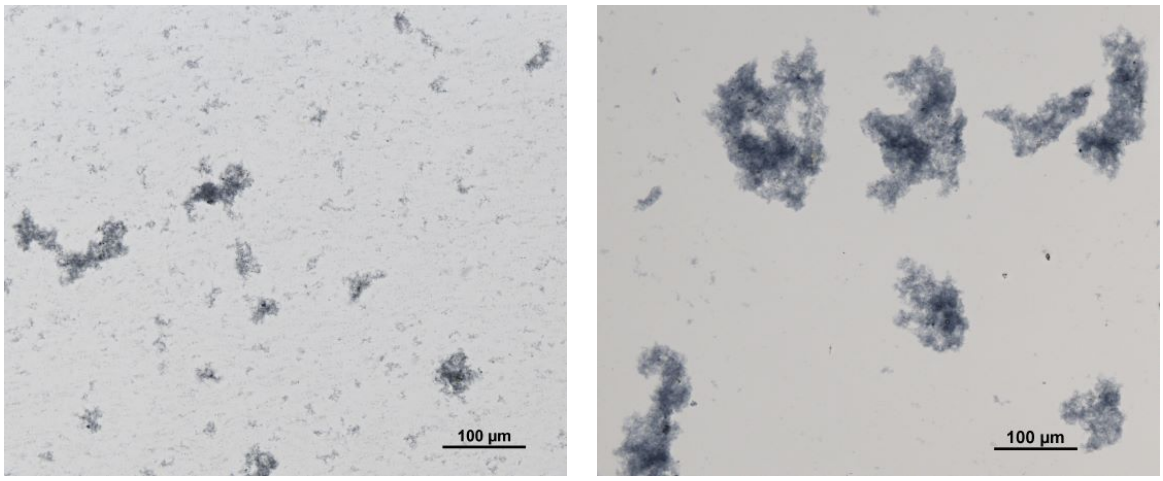
(c) Heat treated insoluble-fraction (non-sieved)

(d) Heat treated insoluble-fraction (sieved)

Figure 4.1: Light micrographs of sieved and non-sieved insoluble-fraction. The heat treated samples were heated in a 95 °C water bath for 30 minutes. Scale bars correspond to 100 µm. Objective/magnification: 10X. Staining: Iodine.

Comparing the sieved with the non-sieved insoluble-fraction shows that the sieving effectively removed the larger husk particles seen in the non-sieved fraction resulting in a flour with higher proportion of starch. Nonetheless, it should be noted that smaller pieces originating from the husk are still present also in the sieved fraction.

The micrographs of the soluble-fraction (figure 4.2) showed no clearly recognisable structural features. But the content did aggregate to some extent, with increased aggregation after heat treatment. The content was weakly stained in blue/grey by Lugol's solution.



(a) Soluble -fraction **(b)** Soluble-fraction, heat treated

Figure 4.2: Light micrographs of soluble-fraction. The heat treated samples were heated in a 95 °C water bath for 30 minutes. Scale bars correspond to 100 µm. Objective/magnification: 10X. Staining: Iodine.

The viscosity of a 1 % (wt) solution of soluble-fraction was also measured as a function of shear rate (measured from 100 to 10 s^{-1}), see Figure 4.3. The results show a shear thinning behaviour indicating that a random coil/rigid polymer structure is present rather than a compact structure which usually results in a viscosity independent of shear rate [22].

Table 4.1 gives the resulting amounts after freeze drying of the different fractions extracted from green lentils. Other studies have, as mentioned in section 2.1.3, reported a starch content of 42g starch per 100g of dry lentils and soluble and insoluble fibre contents in the range of 1-3 (g/100g) and 19-22 (g/100g) respectively. These values indicates that the insoluble-fraction contain mostly starch and insoluble fibres and the soluble-fraction has a weight close to what have been reported as the content of soluble fibre in green lentils.

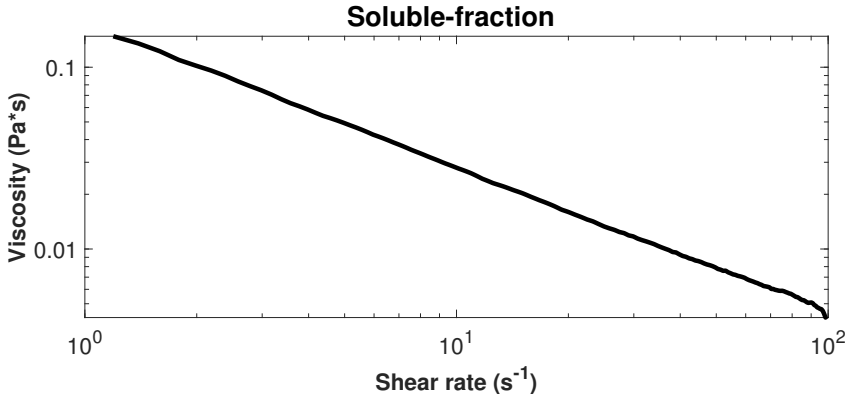


Figure 4.3: Logarithmic plot of the viscosity of a 1 % (wt) solution of soluble-fraction extracted from green lentils.

Extracted fraction	Gram extract per 100g dry lentils
Insoluble-fraction (non-sieved)	68
Insoluble-fraction (sieved)	56
Soluble-fraction	1.9
Protein-fraction	6.0

Table 4.1: Resulting weight of the different fractions extracted from green lentils (*Lens culinaris*) reported as gram per 100 gram of dry lentils.

In summary, the fraction referred to as the *insoluble-fraction* is believed to contain mainly starch and insoluble fibres including larger pieces from the husk and the *soluble-fraction* is believed to mainly contain pectin and other soluble fibres.

4.2 Uniaxial compression

Compression tests were performed to analyse the texture of the gels in terms of fracture stress, fracture strain and Young´s modulus. The effect on these parameters after addition of insoluble-fraction and soluble-fraction was investigated as well as the effect of a change in pH on pure protein and combined protein/insoluble-fraction gels.

In Figure 4.4-4.6 the effect of pH on pure protein gels can be seen. The increase in fracture stress at pH 4.2 for the pure protein gels is in contrast to what has previously been reported for pea protein gels [4]. The pure protein gels at pH 4.2 will be remeasured using a more sensitive load cell suitable for lower stresses to verify this difference. The fracture strain shows an increase closer to the IEP and is in line with previous results [4]. The Young´s modulus shows a slightly different pH dependence than what has been reported [4]. These differences highlights the fact that there are differences between different PPIs due to for example extraction/isolation method and cultivar giving rise to different properties of the pea protein gels.

4.2.1 Insoluble-fraction

Figure 4.4-4.6 also summarises the results from the uniaxial compression tests for gels containing different ratios of insoluble-fraction at different pH. An increase in fracture stress with increased amount of insoluble-fraction can be seen in Figure 4.4 for all pH, and the effect of pH seems to differ compared to the gels containing only PPI. Light microscopy analysis, see section 4.4.1, shows what seems to be starch stabilising the protein network by forming connections between protein aggregates. A higher connectivity in the gel network structure could explain the increase in fracture stress.

The swelling of the starch granules might be another possible explanation for the increased fracture stress after adding insoluble-fraction. The swelling of the granules leads to an increased protein concentration in the surrounding solution. An increased protein concentration will give a stronger gel with a higher fracture stress. This has previously been proposed as an explanation for the increase in G' of whey protein-cassava starch gels [36].

A decrease in fraction stress with increased pH has previously been reported for pea protein gels at a slightly lower concentration [4]. One factor proposed to at least partially explain this is the porosity of the gels. Increased porosity within the protein network leads to a reduction in the fracture surface and hence a decrease in fracture stress values [4]. Increased porosity could also lead to step-wise crack growth due to inhomogeneity on a micro-scale [4].

An increase in pore size (macro pores) was visually observed when comparing prepared protein gels at different pH (see Figure A.2, Appendix A). A reduced pore size can also be observed when insoluble-fraction is added as compared to corresponding gels without the addition (see Figure A.1, Appendix A). However, no clear difference in pore size was observed when increasing the insoluble-fraction concentration.

Munialo et al. (2015) did also observe a change from a fine to a more coarse stranded protein network when approaching the IEP, with the structural transition occurring around pH 3.7 [5]. This gives a less dense network structure with larger inhomogeneous pore sizes and have previously been reported to have a lower fracture stress [5]. The larger defects in coarser gels might be the reason for their lower fracture stress [5].

The decrease in fracture stress with increased pore size and a pH closer to the IEP giving rise to a more coarse stranded protein network is in agreement with the results for the gels with insoluble-fraction added. However, the same trend could not be observed for the gels containing only PPI. This difference in pH dependence for gels with insoluble-fraction added suggest that different types of interactions play larger roles in the network formation after insoluble-fraction is added compared to the pure protein gels.

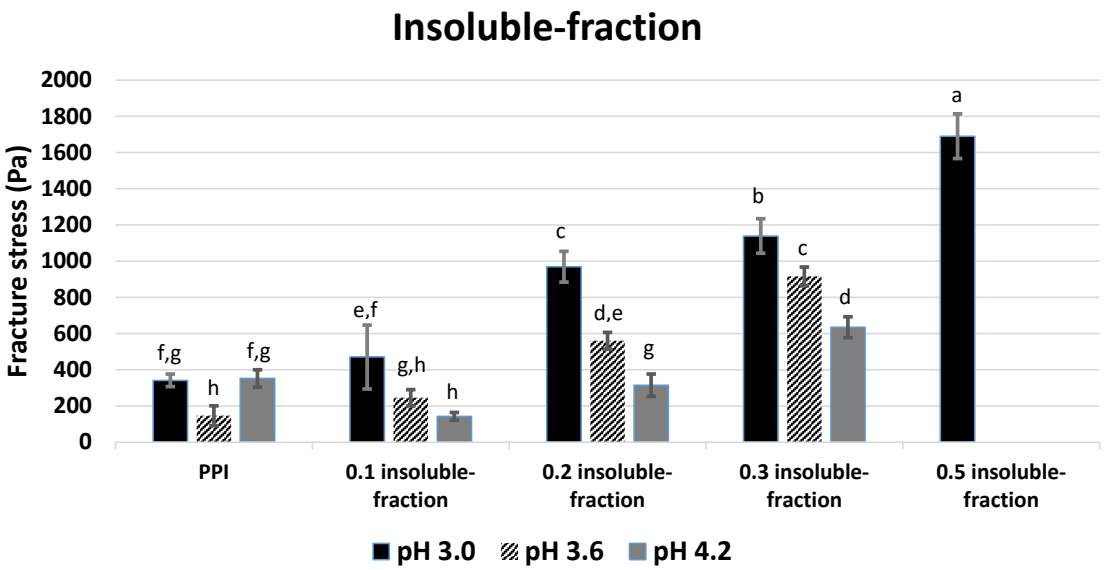


Figure 4.4: Fracture stress of pea protein gels (150mg PPI/mL water) at different pH and insoluble-fraction added to a ratio from 1:10 to 5:10 (insoluble-fraction:PPI). Different letters indicate a statistical difference ($p<0.05$). Error bars represent plus/minus one standard deviation, each calculated from 6-9 replicates.

The results for the fracture strain, Figure 4.5, is less clear and the possible effect of increasing the insoluble-fraction concentration might differ at different pH. The only consistent trend seems to be a lower fracture strain at pH 3.6 as compared to pH 3 and 4.2, regardless of amount of insoluble-fraction added. However, the variations are to large to confirm that there are significant differences between most of the samples. The possible different trends at different pH suggest that there may be several contributions that set the fracture strain of heat induced pea protein gels in more complex systems.

Munialo et al. (2015) observed a large (about 4 times higher) increase in fracture strain at pH 4.2 as compared to lower pH (lowest pH 3) [4]. This was believed to be largely due to the change to a more coarse stranded network resulting in more brittle gels with a lower fracture strain [4]. A similar, but less pronounced, trend can be observed in Figure 4.5 for the pure protein gels but not the gels with insoluble-fraction added. It is possible that an even higher pH as well as more replicates could have shown a similar trend also for gels with insoluble-fraction added. This again indicates a different pH dependence for the gels with insoluble-fraction added as compared to the pure protein gels. The transformation towards a coarse stranded network might also occur later for the PPI used in this study, reducing the effect of the change in pH.

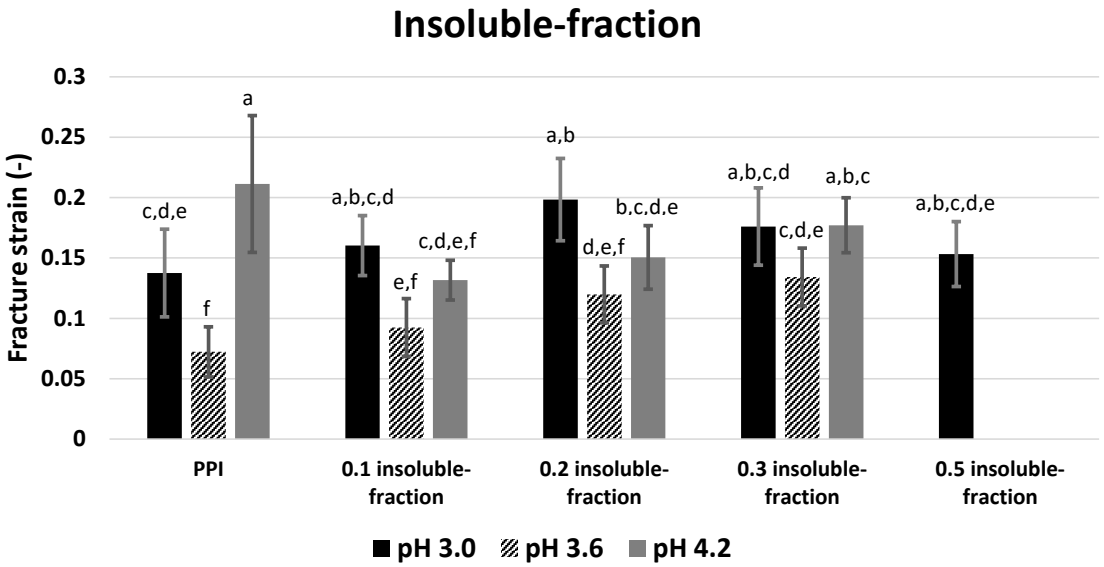


Figure 4.5: Fracture strain of pea protein gels (150mg PPI/mL water) at different pH and insoluble-fraction added to a ratio from 1:10 to 5:10 (insoluble-fraction:PPI). Different letters indicate a statistical difference ($p<0.05$). Error bars represent plus/minus one standard deviation, each calculated from 6-9 replicates.

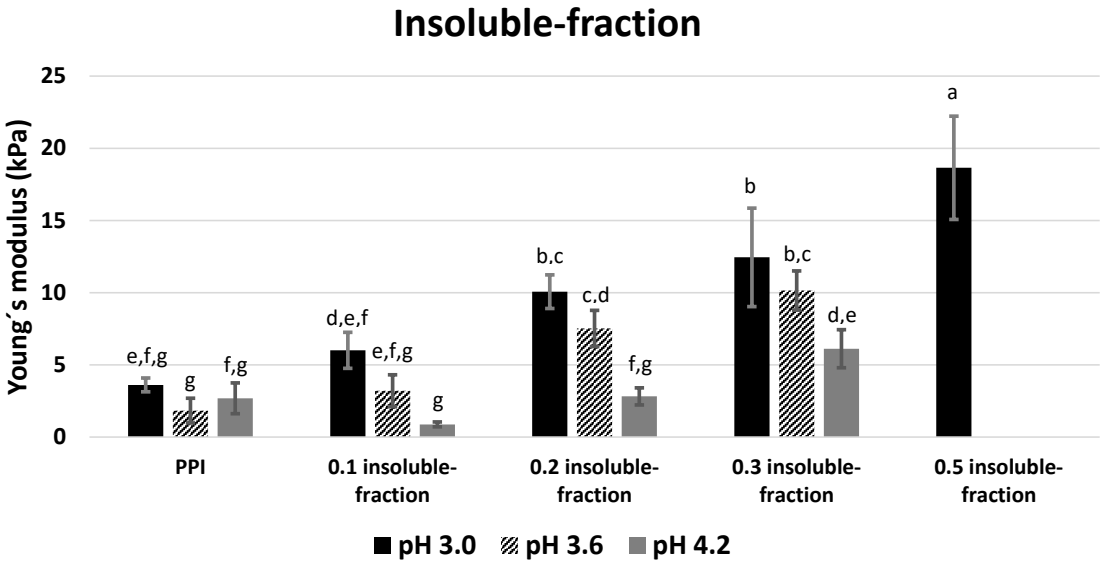


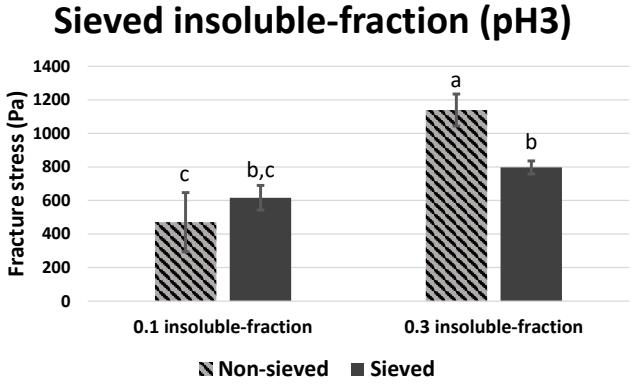
Figure 4.6: Young's modulus of pea protein gels (150mg PPI/mL water) at different pH and insoluble-fraction added to a ratio from 1:10 to 5:10 (insoluble-fraction:PPI). Different letters indicate a statistical difference ($p<0.05$). Error bars represent plus/minus one standard deviation, each calculated from 6-9 replicates.

The Young´s modulus shown in Figure 4.6 shows a similar pattern as the fracture stress in Figure 4.4. An increase with increasing insoluble-fraction added as well as

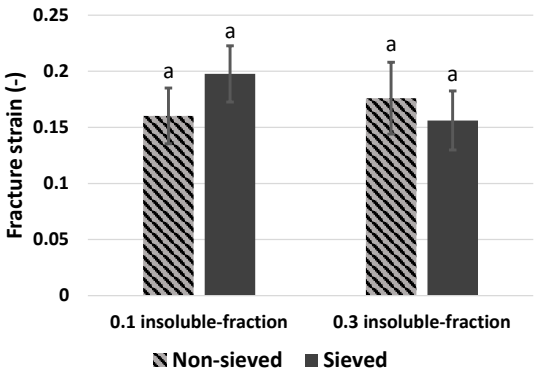
a decrease with increasing pH, again with the exception of the PPI gels at pH 4.2.

The fracture stress, fracture strain and Young´s modulus were measured also for the sieved insoluble-fraction at pH 3 and the results can be seen in Figure 4.7. The decrease in fracture stress observed at the higher concentration could be due to the removal of the larger stiff particle, e.g. husk and cell wall pieces. These particles could serve as reinforcement in the gel structure and their removal resulting in weaker gels.

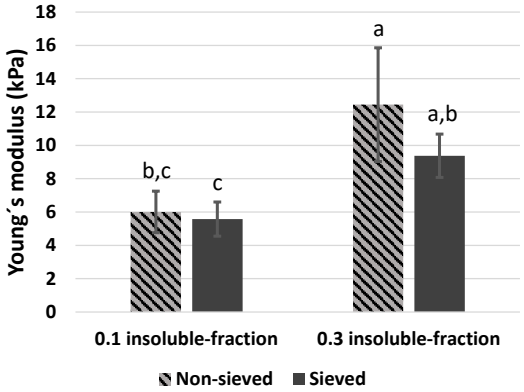
For the fraction strain and Young´s modulus, no significant effect was observed for the sieving of the insoluble-fraction. This was expected since these properties are dominated by the properties of the continuous gel network.



(a) Fracture stress



(b) Fracture strain



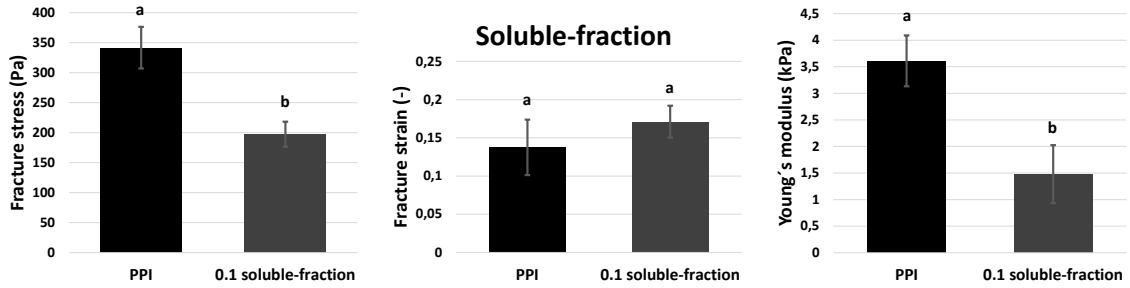
(c) Young´s modulus

Figure 4.7: Fracture stress, fracture strain and Young's modulus of pea protein gels (150mg PPI/mL water) at pH 3 with sieved or non-sieved insoluble lentil fraction added to a ratio of 1:10 and 3:10 (insoluble-fraction:PPI). Different letters indicate a statistical difference ($p<0.05$). Error bars represent plus/minus one standard deviation, each calculated from 5-7 replicates.

4.2.2 Soluble-fraction

A large decrease in fraction stress was observed when adding soluble-fraction at a ratio of 1:10 (soluble-fraction:PPI). Hence, the addition of the soluble-fraction seems to weaken the protein gel network resulting in a lower gel strength. Previous research has shown that both whey protein and pea protein can interact with pectin to form covalent protein-polysaccharide complexes. [60, 61] In order to understand if similar complexes are formed in our gels, and why a weakening of the gels is observed, further research is needed.

No significant difference in fracture strain could be observed, similarly to corresponding addition of insoluble-fraction. The Young´s modulus decreased after addition of soluble-fraction resulting in a less stiff gel.



(a) Fracture stress **(b)** Fracture strain **(c)** Young´s modulus

Figure 4.8: Fracture stress, fracture strain and Young's modulus of pea protein gels (150mg PPI/mL water) at pH 3 with soluble-fraction added to a ratio of 1:10 (soluble-fraction:PPI). Different letters indicate a statistical difference ($p<0.05$). Error bars represent plus/minus one standard deviation, each calculated from 5-7 replicates.

4.3 Dynamic rheology

Dynamic rheology was studied using a DIN bob-cup system for solutions with only PPI and PPI combined with soluble-fraction and insoluble-fraction at pH3. Initially, efforts were also made trying to use a parallel plate system. However, this system was discarded due to what seemed to be a hindered gelation process and large variation in the storage modulus during the cooling part of the temperature ramp. The less pronounced increase of the storage modulus during cooling using the parallel plate system could be partially due to the prevention of evaporation. In both the DIN bob-cup system and when gels were prepared for compression tests, evaporation was allowed whereas it was hindered when using the parallel plate system. The absence of solvent trap and low density oil for the DIN bob-cup system most likely results in an overestimation of the storage modulus as compared to if the evaporation would have been hindered.

For all the samples, the final G′-values were considerably larger in magnitude than the final G″ with tan(δ)-values ranging from 0.18 to 0.22 for all of the gels. This suggests that the gels formed were predominantly elastic.

For the samples containing only PPI, the gelation process can be followed as described earlier in section 2.2.1. First there is an increase in the storage modulus during the heating which can be explained by denaturation of the proteins and interactions between the recently exposed interaction sites. During the holding of the temperature at 95°C the G′ stays relatively constant with a possible small increase of the G′ for some of the samples. During the cooling phase a significant increase in the storage modulus is observed as the gel is further strengthened due to hydrogen bonding and other short-range interactions. As the cooling continues, more and more of these bindings can withstand the previously to high molecular kinetic energy, hence increased number of interactions and an increase in G′.

4.3.1 Insoluble-fraction

From the compression results as well as visual and practical handling of the gels, where a clear difference in gel strength and gel stiffness could be observed, an increase in G′ was expected with addition of insoluble-fraction. This can also be observed in Figure 4.9.

The increase in the storage modulus with addition of insoluble-fraction is believed to be an effect of the starch mainly. Both by increasing the connectivity due to the starch forming connections between the protein network, as will be seen in section 4.4.2, as well as the swelling of the starch granules resulting in a higher protein concentration in the surrounding solution. The samples with insoluble-fraction added shows a similar development of the storage modulus as the pure PPI gels with some small differences further discussed in connection to Figure 4.10.

Table 4.2 and 4.3 gives an overview of the dynamic rheology measurements. The results are divided into two separated groups (one including the insoluble-fraction and one the soluble-fraction measurements) due to a shift towards lower G′-values. The main reason for the decrease is believed to be a change of pipette resulting in a lower sample volume. All measurements, except for soluble-fraction added at a ratio of 0.2 (soluble-fraction:protein), were run with at least two replicates. However, due to some complications with the heating system only 1 replicate is used for plotting the storage modulus for some of the samples.

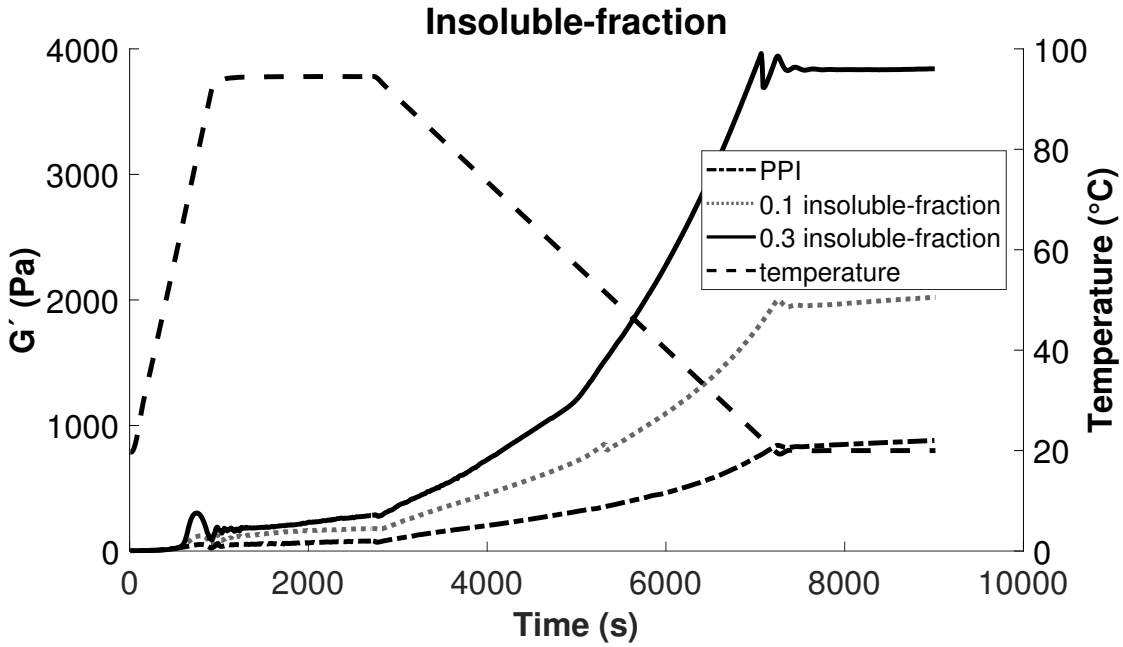


Figure 4.9: G′ as a function of time during temperature change simulating the gelation process of pea protein gels (150 mg PPI/mL water) with insoluble-fraction added to a ratio of 1:10 and 3:10 (insoluble-fraction:PPI).

One replicate for each insoluble-fraction ratio was also run using the new pipette with lower volume (results not shown). These results replicated the pattern shown for the earlier measurement, but shifted towards lower G′-values. The final storage modulus for the lowest concentration (insoluble-fraction to PPI ratio of 0.1) of insoluble-fraction was still higher than the highest concentration (soluble-fraction to PPI ratio of 0.2) of soluble-fraction.

Sample	**G′(Pa)**	**Gel point***	**Replicates**
PPI	1038 ± 0.4	25.50 ± 0.84 (3)	2
0.1 insoluble-fraction	1970 ± 51	26.26 ± 1.56 (4)	2
0.3 insoluble-fraction	3624 ± 217	28.92 ± 0.682 (3)	2

Table 4.2: Storage modulus at different points during the gelation process, including standard deviation (plus/minus one standard deviation) of pea protein gels (150 mg PPI/mL water) with insoluble-fraction added to a ratio of 1:10 and 3:10 (insoluble-fraction:PPI). Numbers within parenthesis indicate different number of replicates used to estimate the gel point than stated in the "Replicates"-column. *Gel point defined as the temperature at which tan(δ) goes below 0.5.

Due to the non-existing crossover of the storage and loss modulus for most of the samples and the fact that no clear start of the increase in storage modulus could be defined, an alternative way was used to compare the samples in terms of their gel point. Instead of the crossover (tan(δ)=1), an alternative value of tan(δ) equal to 0.5 was used instead. This represents the point at which the storage modulus

reaches twice the value of the loss modulus. This value was chosen to include as many of the replicates as possible without mowing to far away from the original definition. The results are reported in table 4.2.

Some difference in the gelation process can be observed when adding insoluble-fraction. This is more clearly visualised when normalising the data according to equation 4.1, where t* is the time corresponding to the end of the cooling phase and start of the holding time at 20 °C, as can be seen in Figure 4.10. The normalising of the data makes it possible to follow the development of the gel formation in some more detail.

$$G'(t)^* = \frac{G'(t)}{G'(t = t^*)} \tag{4.1}$$

The main difference after addition of insoluble-fraction can be observed in the first part of the figure during the heating. This is better visualised in the zoomed in figures in appendix A.3. When the temperature reaches about 60°C the storage modulus of the samples including insoluble-fraction starts to increases faster than the pure protein samples, and around 70°C the maximum increase in G′ is reached. These two temperatures corresponds very well with the temperatures of the start and maximum of gelatinization of lentil starch reported from DSC measurements [23, 24]. Hence this difference in the gelation process after addition of insoluble-fraction is believed to be an effect of the presence starch.

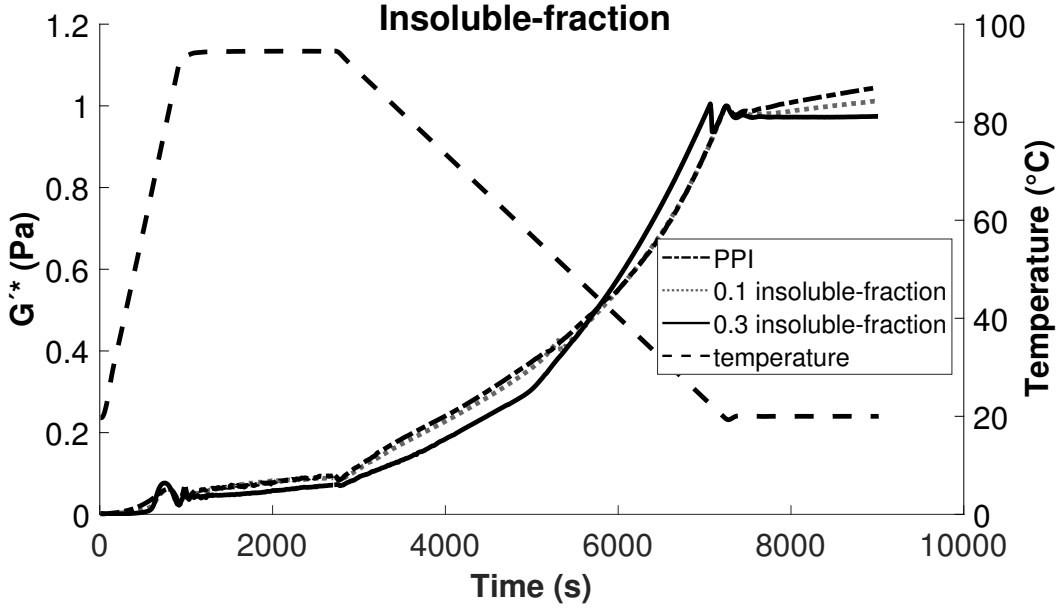


Figure 4.10: Normalised G′ as a function of time during temperature change simulating the gelation process of pea protein gels (150 mg PPI/mL water) with insoluble-fraction added to a ratio of 1:10 and 3:10 (insoluble-fraction:PPI). Normalised according to equation 4.1.

There is a slight difference in the development of the storage modulus also during cooling. As the temperature goes below 60°C there is a faster increase of the storage modulus for the samples with insoluble-fraction added. The difference can clearly be observed for the higher concentration of insoluble-fraction but less clearly for the lower concentration. This change could not be seen for the pure protein samples and is believed to be due to the setback and gelling of the cooked starch occurring when the amylose starts to realign and form a network.

4.3.2 Soluble-fraction

The addition of soluble-fraction resulted in a slight increase of the storage modulus for the final G′-value, see Figure 4.11. But the increase is smaller than after addition of insoluble-fraction. The different values for the pure protein gels (PPI) in Figure 4.11 as compared to Figure 4.9 is believed to be mainly due to a slightly smaller volume.

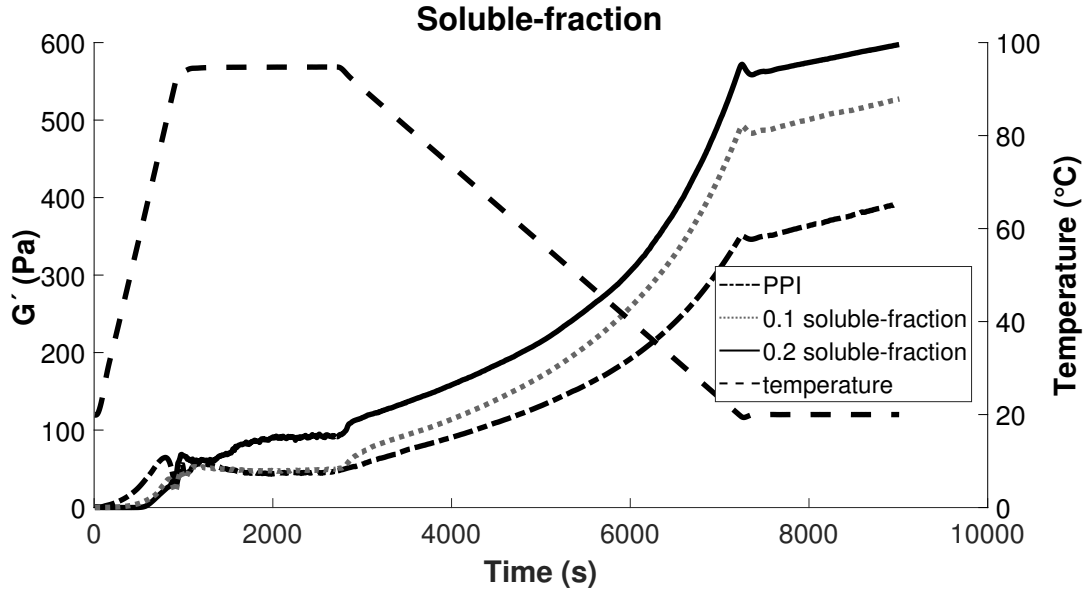


Figure 4.11: G′ as a function of time during temperature change simulating the gelation process of pea protein gels (150 mg PPI/mL water) with soluble-fraction added to a ratio of 1:10 and 2:10 (soluble-fraction:PPI).

The development of the storage modulus and the kinetics of the gel formation can again be followed in slightly more detail in Figure 4.12 for the soluble-fraction. Difference from the gelation of the pure protein gels can be noted at the beginning during heating with a delayed and slower increase in G′. This part of the graph is again better visualised in the zoomed in figures in appendix A.3. For the higher concentration of soluble-fraction this delayed gelation is very clear and the increase in storage modulus does not occur until a temperature above 60°C is reached. During the cooling part and resting at 20 °C the development is similar to the only protein

solution.

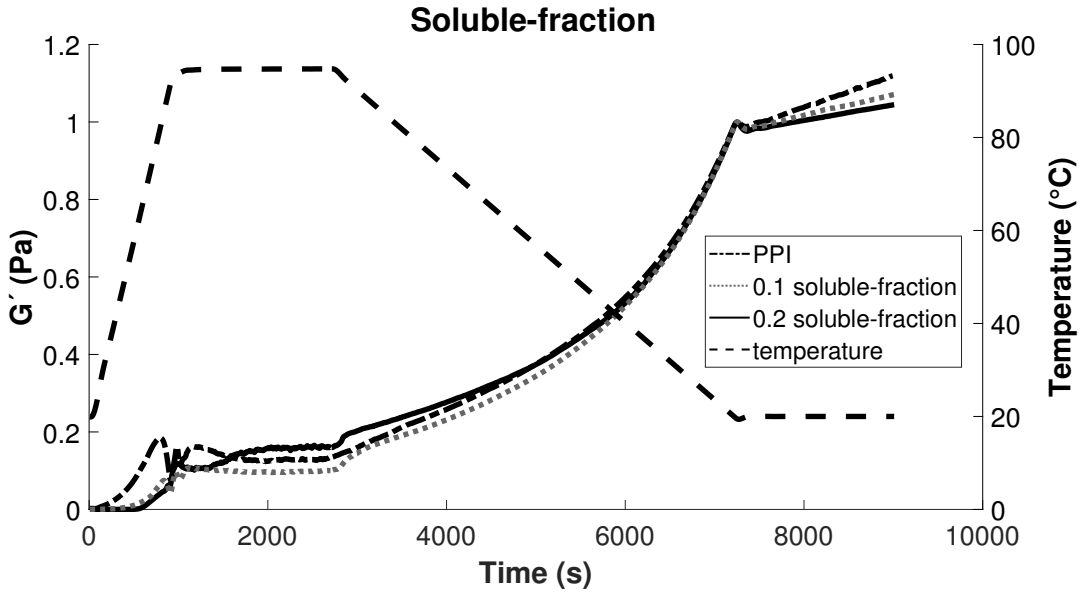


Figure 4.12: Normalised G′ as a function of time during temperature change simulating the gelation process of pea protein gels (150 mg PPI/mL water) with soluble-fraction added to a ratio of 1:10 and 2:10 (soluble-fraction:PPI). Normalised according to equation 4.1.

For the 0.1 soluble-fraction solution the gelling point (defined by storage and loss modulus crossover) occurred around 29°C. For the 0.2 soluble-fraction, a gelling point could be defined from the crossover of the storage and loss modulus as 58.6°C. For all the other solutions, the storage modulus was larger than the loss modulus already at the starting temperature (20°C). This further shows how the gelation is delayed when the soluble-fraction is added. Since no crossover was observed for the other gels, the gel point is reported also using the alternative definition (tan(δ)=0.5) in table 4.3.

Sample	G′(Pa)	Gel point*	Number of replicates
PPI	393 ± 85	25.50 ± 0.84 (3 replicates)	2
0.1 soluble-fraction	527 ± 55	43.27 ± 4.98	3
0.2 soluble-fraction	598	61.35	1

Table 4.3: Storage modulus and gel point, including standard deviation (plus/minus one standard deviation), of pea protein gels (150mg PPI/mL water) with soluble-fraction added to a ratio of 1:10 and 2:10 (soluble-fraction:PPI). Where no standard deviation is reported, there was only one replicate. Different letters indicate a statistical difference ($p<0.05$). *Gel point defined as the temperature at which tan(δ) goes below 0.5.

4.3.3 Summary of rheology

Table A.1 summarises the results from the uniaxial compression tests and dynamic rheology. The general effect of addition of soluble lentil fibre- and insoluble lentil-fraction are shown.

	G′	Fraction stress	Fraction strain	Young´s modulus
Insoluble-fraction	+	+	+/-	+
Soluble-fraction	+	-	+/-	-
Increased pH	nd	+/-	+/-	+/-
Increased pH*	nd	-	+/-	-

Table 4.4: Summary of the effect of pH change and addition of soluble or insoluble lentil fraction on rheological properties of pea protein gels. Symbols clarification: "+":increase, "-": decrease, "+/-": not clear, "nd": not determined. * For combined insoluble-fraction/PPI-gels.

4.4 Microscopy

Using microscopy, the starch and protein structures could be visualised in the gels. The structure of the protein aggregates and their interaction with the starch could be seen using LM and with CLSM the structure of the protein network could be confirmed on fresh gels.

4.4.1 Light microscopy

Light microscopy was used to obtain an overview of the microstructure for the different gels. Using Iodine, protein and starch could be stained in yellow and purple respectively. Figures are chosen to be as representative as possible for the general protein network, and in most cases no larger structures, such as pores or pieces of husk, are included in the figures. There were also some differences in the density of the protein network within sections obtained from cryo-sectioning. This was believed to occur mainly due to the freezing and cutting of the samples. Micrographs were chosen from similar regions for each sample.

For most of the gels, the protein network seems to consist of larger protein aggregates (10-100 µm) and smaller and less distinct ones filling the rest of the volume together with empty areas (mainly water). The less distinct network seems to be more homogeneous at pH 3, for the pure protein gels, as compared to the other pH, see Figure 4.13 (a, c, e). The reason for the less homogeneous network formed at the higher pH might be due to a pH closer to the isoelectric point. Closer to the isoelectric point, the overall charge of the proteins will be close to zero and hence the proteins will aggregate easier. The difference in charge could lead to a different aggregation behaviour resulting in a difference in microstructure.

A previous study have shown that there is a change in microstructure at pH close to the isoelectric point as compared to further away [4]. They reported a change

of the pea protein network from a finer towards a coarser network around pH 3.7. This change was however observed at a smaller length scale (100 nm). It is possible that this change in network structure also effects the network structure on a larger length-scale, and that is what can be observed in Figure 4.13.

4.4.2 Insoluble-fraction

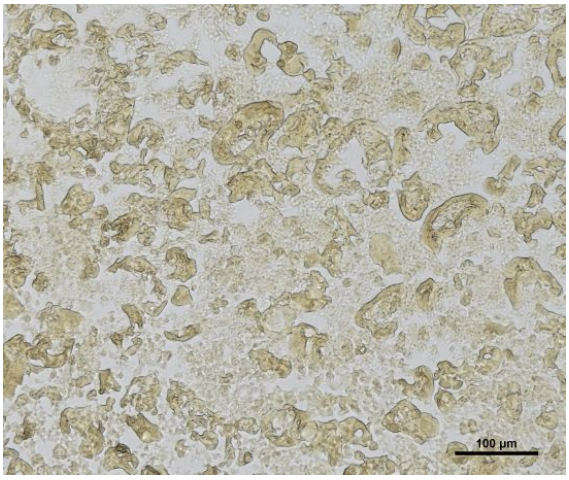
Figure 4.13 shows light micrographs of pea protein gels with and without insoluble-fraction added. A more dense protein network (yellow) form at pH 3 compared to pH 3.6 and pH 4.2. The way by which the starch (dark blue/purple) interacts with the pea protein network seems however to be similar at all investigated pH. At all pH the starch forms connections bridging between the protein aggregates. This strengthening of the protein network by the starch can be seen more easily in Figure 4.14 which shows the same gels at a higher magnification.

In Figure 4.14 it can also be observed that part of the starch is located in the smaller and less distinct protein aggregates. Possibly forming similar connections between protein aggregates as well as between different parts of the same aggregate, but on a smaller length scale.

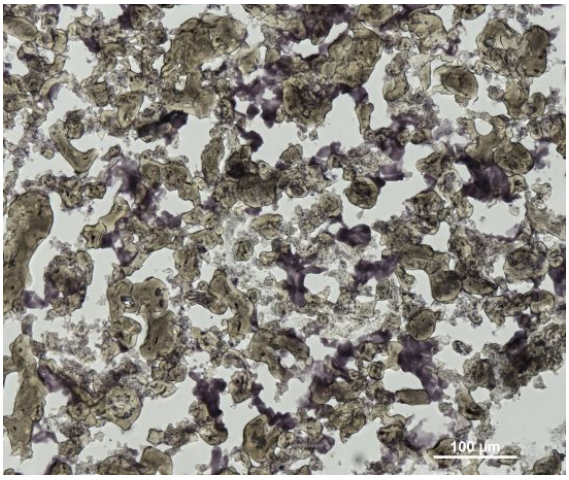
Some of the larger areas with starch, forming connections between the protein aggregates, does to some extent resemble the granule ghosts seen in Appendix A.2, Figure A.3. The presence of protein inhibiting gelation by reducing water availability as well as the absence of shear during the heating process could be the reason for these granule ghosts. Overall, some of the starch seems to be concentrated in these remaining structures of the granules and others aggregating to form similarly sized or smaller structures.

There is also a change in the microstructure when insoluble-fraction is added. The somewhat homogeneous network seen around the larger protein aggregates mainly at pH 3 for the PPI gels seems to be disrupted when adding insoluble-fraction. This change occurs also for the higher pH, but is less distinct due to the change in pH already affecting the structure in a similar way. The reason for this change in microstructure might be the starch, but could also be due to the other contents of the insoluble-fraction, such as fibre.

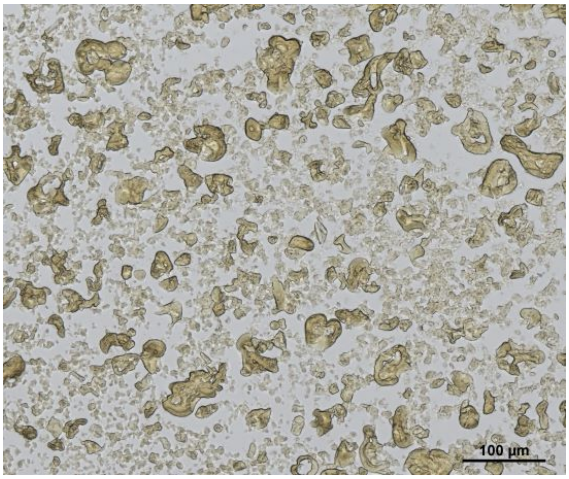
The larger empty areas present only in the gels with insoluble-fraction added could be a result of the swollen starch granules. During the heating process, the granules will swell and when the temperature is high enough the protein network start to form a network around them. If the granules do not break and disintegrate before the main structure of the protein network is formed, this could possibly create larger areas filled with water when the granules eventually disintegrate.



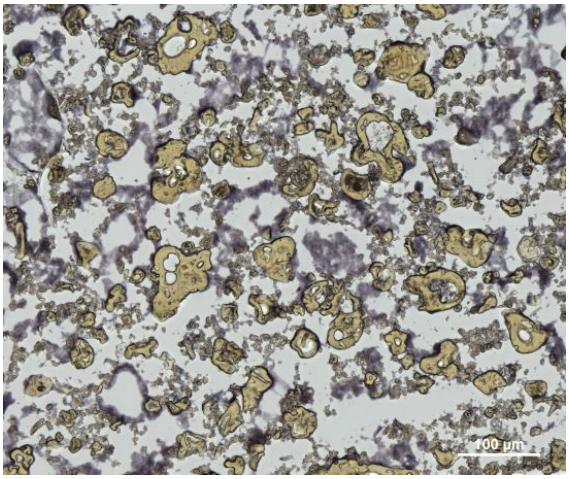
(a) pH 3.0; PPI, 10X



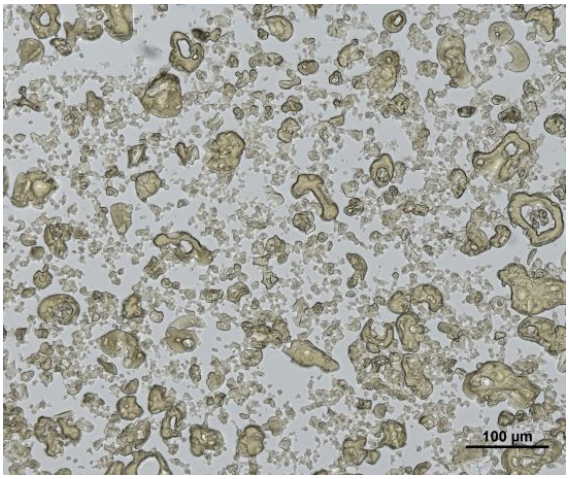
(b) pH 3.0; 0.3 insoluble-fraction, 10X



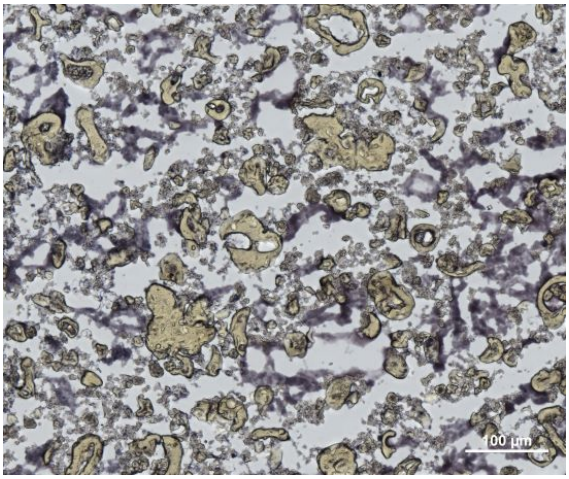
(c) pH 3.6; PPI, 10X



(d) pH 3.6; 0.3 insoluble-fraction, 10X

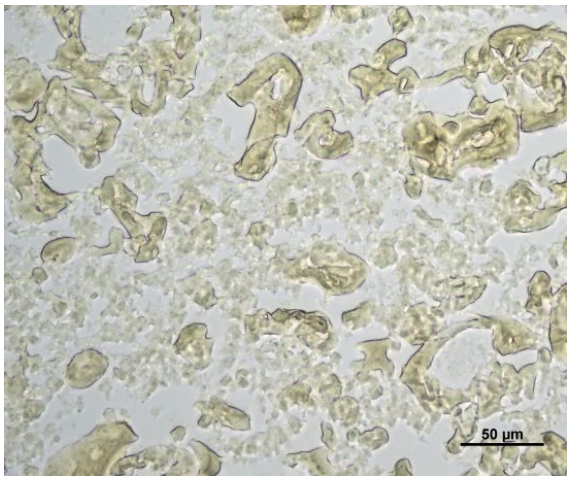


(e) pH 4.2; PPI, 10X

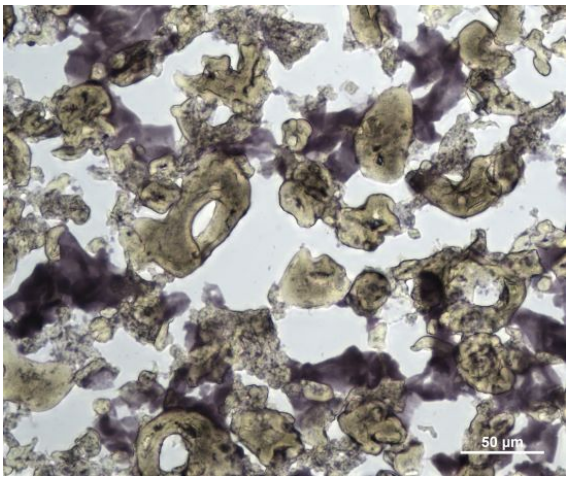


(f) pH 4.2; 0.3 insoluble-fraction, 10X

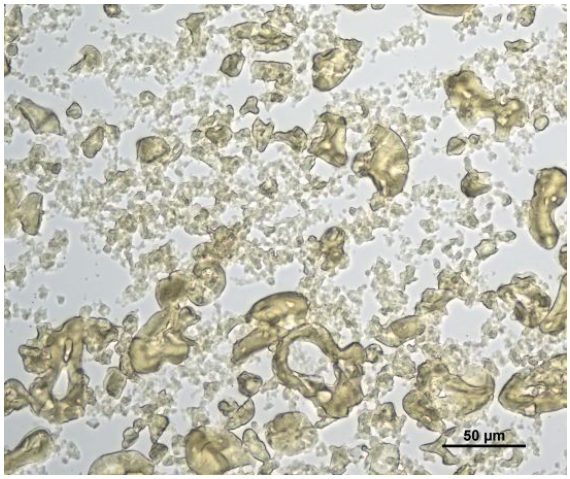
Figure 4.13: Light micrographs of pea protein gels (150 mg PPI/mL water) at different pH and with or without insoluble-fraction added to a ratio of 3:10 (insoluble-fraction:PPI). Scale bars correspond to 100 µm. Objective/magnification: 10X. Staining: Iodine.



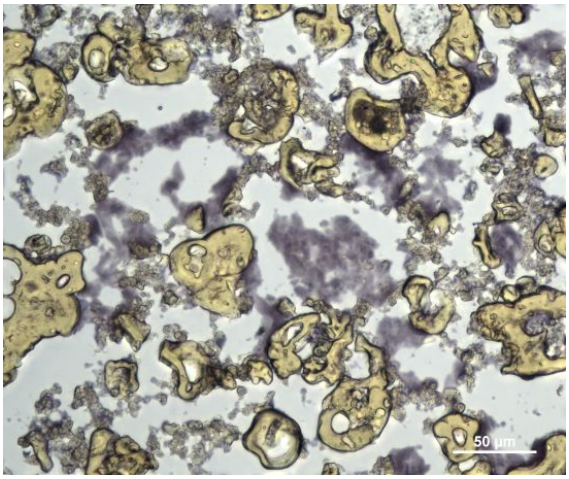
(a) pH 3.0; PPI, 20X



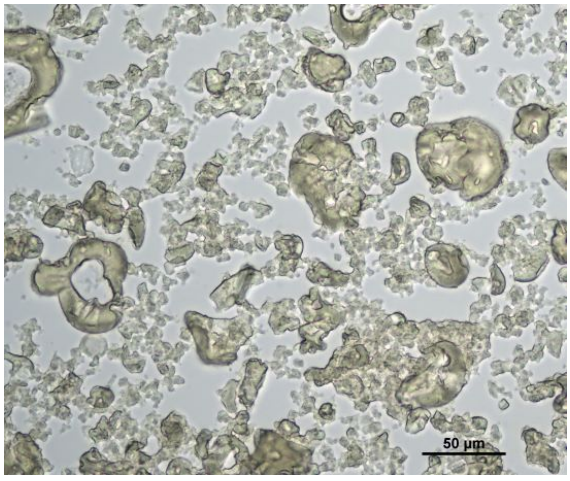
(b) pH 3.0; 0.3 insoluble-fraction, 20X



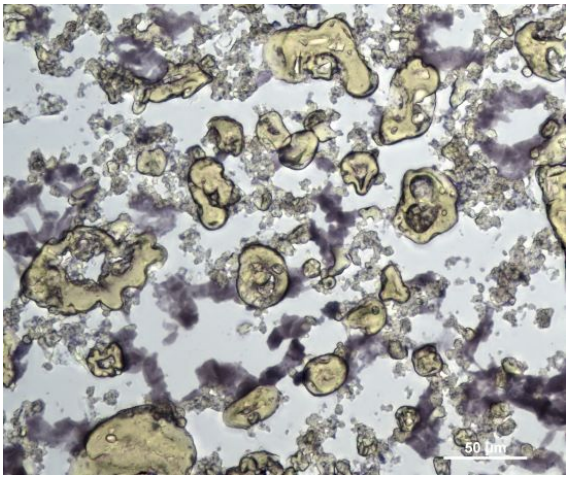
(c) pH 3.6; PPI, 20X



(d) pH 3.6; 0.3 insoluble-fraction, 20X



(e) pH 4.2; PPI, 20X



(f) pH 4.2; 0.3 insoluble-fraction, 20X

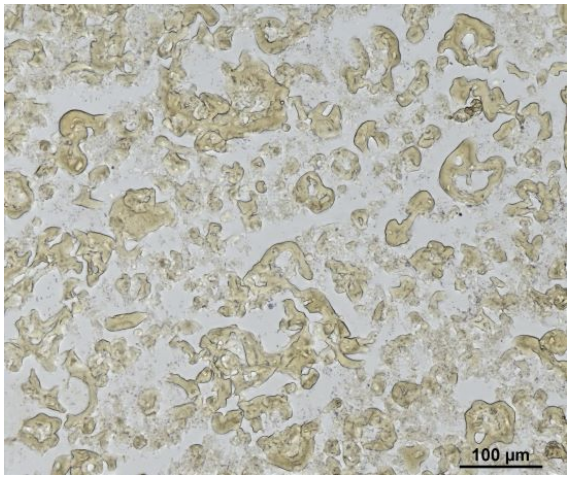
Figure 4.14: Light micrographs of pea protein gels (150 mg PPI/mL water) at different pH and with or without insoluble-fraction added to a ratio of 3:10 (insoluble-fraction:PPI). Scale bars correspond to 50 µm. Objective/magnification: 20X. Staining: Iodine.

The connections formed by the starch between the protein aggregates is believed to be part of the explanation for the change in rheological properties observed when including insoluble-fraction in the gels. A higher connectivity in the gel network structure can explain both the increase in fracture stress, Young´s modulus and G′. A higher connectivity can give a more solid like material with a higher ability to store energy within the structure rather than dissipating it as heat, resulting in a higher strength and storage modulus of the gel [4].

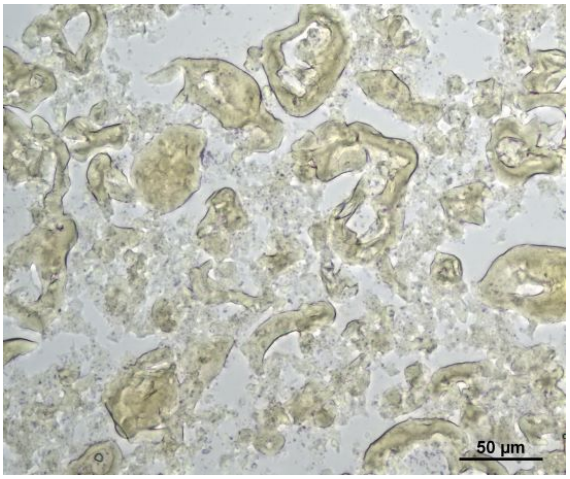
4.4.3 Soluble-fraction

In Figure 4.15 LM micrographs obtained are shown for gels including soluble-fraction. The protein network formed seems to resemble that of only protein at the same pH, with some slight differences. The effect seen when adding starch (disappearance of the more homogeneous network formed by the smaller protein aggregates) cannot clearly be seen when adding soluble-fraction, even if it seems to be slightly changed. This is better visible in the non-stained LM micrographs in appendix A.4.

Since the fibres are not stained by iodine very clearly, it is difficult to say where they end up in the network. However, some small regions/dots (purple) could be observed, see Figure 4.15 (b).



(a) pH 3.0; 0.1 soluble-fraction, 10X



(b) pH 3.0; 0.1 soluble-fraction, 20X

Figure 4.15: Light micrographs of pea protein gels at pH 3 and with soluble-fraction added in a ratio of 1:10 (soluble-fraction:PPI). Scale bars correspond to 100 (a) and 50 µm (b). Objective/magnification: 10X (a) and 20X (b). Staining: Iodine.

When staining a solution containing only the soluble-fraction using iodine, it could be observed in blue, i.e. the fibre or something in the soluble-fraction is stained by the iodine. This indicates that the small blue dots are actually the soluble-fraction.

They also seem to be present only in the less distinct, smaller and almost continuous protein aggregates rather than the larger ones.

4.4.4 Confocal laser scanning microscopy

Gels were also investigated using CLSM. For the CLSM, fresh gels were used for analysis. The images obtained from CLSM showed protein aggregates similar to these observed using light microscopy. For some of the samples a small difference in the shape of the larger aggregates could be noted. A difference in which the larger aggregates in CLSM micrographs having slightly more rounded shapes than the corresponding aggregates in the micrographs obtained from LM. But overall, the general structure with larger aggregates together with smaller ones in between, was comparable for the two microscopy techniques.

Figure A.9 and A.10 in Appendix A.5 shows the micrographs of a pea protein gel (150 mg PPI/mL water) at pH 3 in which the observed protein aggregates are comparable to the corresponding ones observed in Figure 4.13 and 4.14. Since the CLSM micrographs were obtained from gels without any freezing, freeze sectioning or other preparation other than the addition of rhodamin and mounting of samples, this indicates that the preparation for the light microscopy does not greatly alter the structure of the gels.

4.5 Differential scanning calorimetry

For the DSC measurements, a peak corresponding to the denaturation of the protein was expected at around 85-95 °C. *Shand et al. (2017)* reported a denaturation temperature of 85.1°C using similar concentrations (10% (w/w)), volume (10-15mg) and heating rate (10 °C/min) [18]. However, no clear transition temperature was observed for pea protein solutions (150 mg/mL) at pH 3 nor pH 7, see Figure 4.16. The reason for this could be that the protein was already denatured during the production process. The peak might also have been too wide and non-distinct to be observed.

A possible reason for the absence of a denaturation peak could be that the protein was already denatured during the production. *Shand et al. (2007)* compared commercial pea and soy protein isolates with laboratory prepared ones (alkaline extraction (pH 8.5) followed by precipitation (pH 4.5) and freeze drying) [18]. By comparing the commercial and laboratory prepared isolates they could conclude that the commercially obtained isolates was most likely denatured leading to absence of endothermic peaks in DSC measurements.

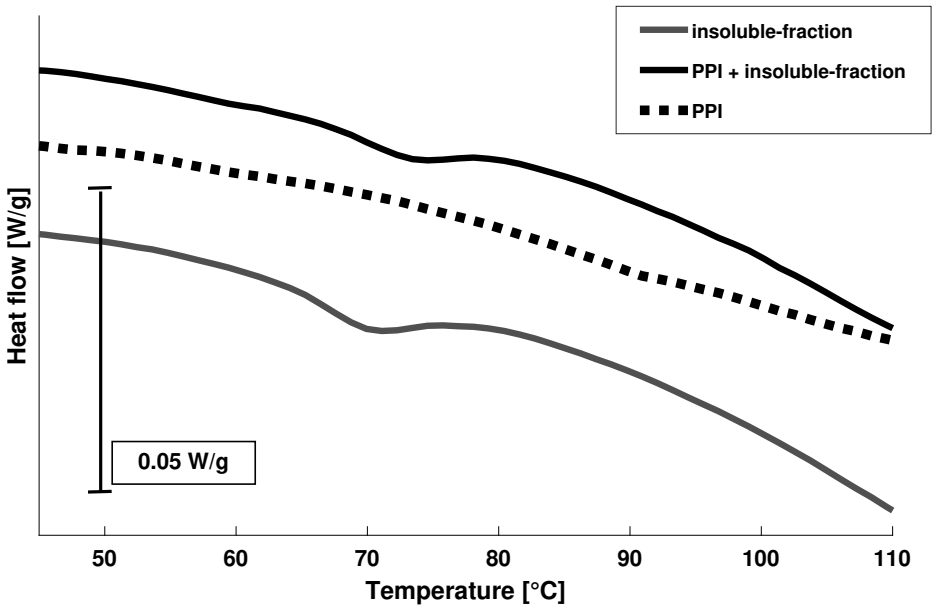


Figure 4.16: DSC measurements for PPI (150mg PPI/mL water), insoluble-fraction (45mg insoluble-fibre/mL water) and insoluble-fraction/PPI solution (45mg insoluble-fibre + 150mg PPI/mL water). Heat flow is reported in units of Watt per gram solution.

Previous studies on pea protein have also reported the missing of a distinct peak at pH 3 (while still observing a peak at higher pH (5, 7 and 9)) [5]. This is believed to be due to denaturation of the protein at low pH and explains the fact that no peak was observed for the samples containing only protein at pH3. However this cannot be the full explanation since no peak was observed at pH 7 either, but further strengthens the theory that denaturation is the cause for the absence of clear peaks.

An already denatured protein might also be part of the reason for the early increase of the G' and why no gel point is observed. Typically, a large increase of the storage modulus is clearly observed around the denaturation temperature of the pea protein during heating, as can be seen in *Sun et al. (2010)* [31]. In Figure A.4-A.5 appendix A.3, it can be seen that the storage modulus showed a exponential increase already from low temperatures rather than staying approximately constant until the expected denaturation temperature around 85°C.

Lower protein concentrations were also tested without success. It was hypothesised that high concentration might lead to protein-protein interactions resulting in a less distinct peak.

For the protein solution with insoluble-fraction added, a peak could be observed around 73.3°C. A similar peak was observed in a solution of only insoluble-fraction, without protein, at a slightly lower temperature, i.e. a small temperature shift was observed, see table 4.5. This transition temperature most likely corresponds to the

gelatinization of the starch present in the insoluble-fraction (literature values for lentil starch: peak: 69.32, onset: 61.56 [24]). The shift in temperature between the solution with only insoluble-fraction and the one also containing protein indicates that the protein effects the starch gelation process. Studies have shown that protein can shift the gelatinization of starch towards higher temperatures. Whey and soy protein have shown to increase the gelatinization temperature of corn starch [62, 63]. This is hypothesised to be a result of the protein lowering the water mobility to the starch granules [62]. The not very sharp shape of the peak indicates a more amorphous and less crystalline structure of the starch granules, but might also be due to the experimental setup and procedure.

The temperature of the observed starch gelatinization peak matches well with the drastic increase in G' in the sample with insoluble-fraction compared to the one without, as seen in Figure 4.10. This further points to the idea that it is the starch that strengthens the pea protein network.

Content	**Onset (°C)**	**Peak (°C)**	**Number of replicates**
Insoluble-fraction only	62.37±0.56	69.94±0.01	2
0.3 insoluble-fraction	67.05±1.32	73.29±0.35	4
PPI	-	-	-

Table 4.5: Gelatinization and transition temperatures (peak and onset) of lentil starch in PPI (150mg PPI/mL water), insoluble-fraction (45mg insoluble-fibre/mL water) and insoluble-fraction/PPI solution (45mg insoluble-fibre + 150mg PPI/mL water). Data obtained from DSC measurements with a heating rate of 10 °C/min.

4.6 Moisture loss

Efforts were made trying to measure moisture loss (ML) by comparing the weight of the gels before and after centrifuging. However, most of the gels were to weak to maintain their structure even at the lowest rotational speed (500 rpm, 42g), making the measurements somewhat irrelevant for the actual gels. The breakage of the gel structure could be observed as the gels were compressed, cracked and some almost forming a paste after being centrifuged.

However, the limited results that was still obtained (see appendix A.6) indicated that the moisture loss increased with increased pH in the measured pH region. Also addition of insoluble-fraction seemed to decrease the moisture loss of the gels. The increase in moisture loss with increased pH can possibly be explained by the change in the network structure, from a finer stranded to a more coarse stranded, seen in previous studies around pH 3.7. In a more coarse stranded network the ability to trap the water might be reduced due to fewer but larger cavities allowing for the water to move more freely.

The possibly reduced water loss by gels after addition of insoluble-fraction could be due to its stabilising and gel strengthening effects. A more stable protein/starch network has a higher ability to retain its structure. Thus entrapped water might be less likely to break the structure trying to escape during the increased pressure experienced while centrifuging.

The gel strength and a more stable protein network could be the explanation for the increased moisture loss with increased pH as well. Increased pH from 3 to 3.6 or 4.2 resulted in a decrease in fracture stress for gels with a 0.3 ratio of insoluble-fraction to PPI. The moisture loss measurements indicates an increased moisture loss with the same increase in pH.

Thinner gels could possibly have solved the issue with deformation and breakage of the gels during centrifuging. Another solution could have been to keep the gels in the glass cylinders, replacing the rubber bottom with a filter paper and placing it directly on the nylon net. This would have supported the structure of the gel without hindering the transfer of water in the direction of the centrifugal force.

5
Conclusion

Insoluble- and soluble-fraction were extracted from green lentils and their effect on the rheology and microstructure of heat induced pea protein gels was studied. Addition of insoluble-fraction increases the fracture stress and Young´s modulus resulting in stronger and stiffer gels. It also increases the storage modulus and the ability to store energy within the structure of the gel. The effects of the insoluble-fraction seems strongly related to its starch content. No clear trends could be observed for the fracture strain.

Addition of soluble-fraction has an effect mainly on the fracture stress and Young´s modulus, which are both significantly reduced upon addition. The storage modulus is increased despite but the gelation is delayed.

Light microscopy revealed that the starch present in the insoluble-fraction forms connection between the larger protein aggregates. There is also a change in the microstructure (on a 100 µm-scale) after the addition of both insoluble- and soluble-fraction. The change in microstructure and formation of a combined protein-starch network might be part of the explanation for changed textural and rheological properties.

A change in pH has an effect on the fracture stress, Young´s modulus and microstructure. The effect of a change in pH seems to differ in gels produced from PPI only and gels with insoluble-fraction added. A pH closer to the IEP of pea protein resulted in a slightly different microstructure.

Overall, increased knowledge of the effect of starch and fibre rich lentil fractions on pea protein gelation can serve helpful in tailoring the textural properties of new and existing plant based foods.

5.1 Further studies

There is still plenty of research to be done on pea protein gelation. Few studies have been conducted on systems containing not only pea protein isolate. Interesting systems could include different starch or fibre rich fractions produced as by-products in food industry. The interaction and effect of other polysaccharides and thickeners such as xhantan gum and agar agar could also be of interest for production of protein rich desserts.

Regarding pea protein-starch gels, the transition from a continuous protein network to a starch network and how it affect rheological properties is another interesting area of research. These high starch-low protein gels might have not only different nutritional properties but also a different texture and applications. Investigating the effect of refined starch could further strengthen the fact that the observed changes in rheological properties and microstructure found in this study is due to the starch present in the insoluble-fraction.

For the effect of fibre on pea protein gelation, refined and characterised fibres with different properties might give more clear results. Together with immunolabelling or other staining techniques the fibres can be localised within the gels to give a better understanding of how they interact with the protein network. Examples of fibres include β-glucan and arabinoxylan which are both found in oats and can both be labelled using immunolabelling.

For both the fibre and starch additions, scanning electron microscopy (SEM) could give more information on the network structure. It could give further insight on the location of the starch and fibre as well as their effect on the protein network.

Chemical analysis of the lentil-fractions used in this study will be performed and hopefully give more insight on why these fractions effect the gelation in the way they do.

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A

Appendix

A.1 Porosity

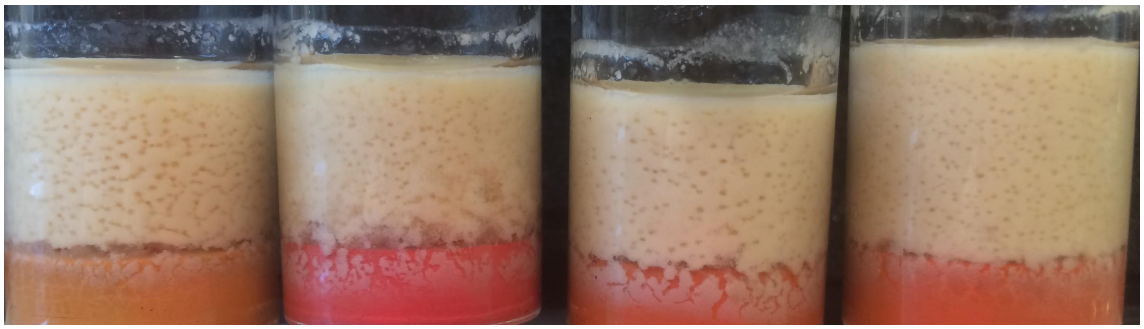


Figure A.1: Pea protein gels (150mg PPI/mL water) at pH 4.2 without and with insoluble-fraction added. Ratio of insoluble-fraction to PPI added, from left to right, 0, 0.1, 0.2 and 0.3 (gram insoluble-fraction per gram PPI).

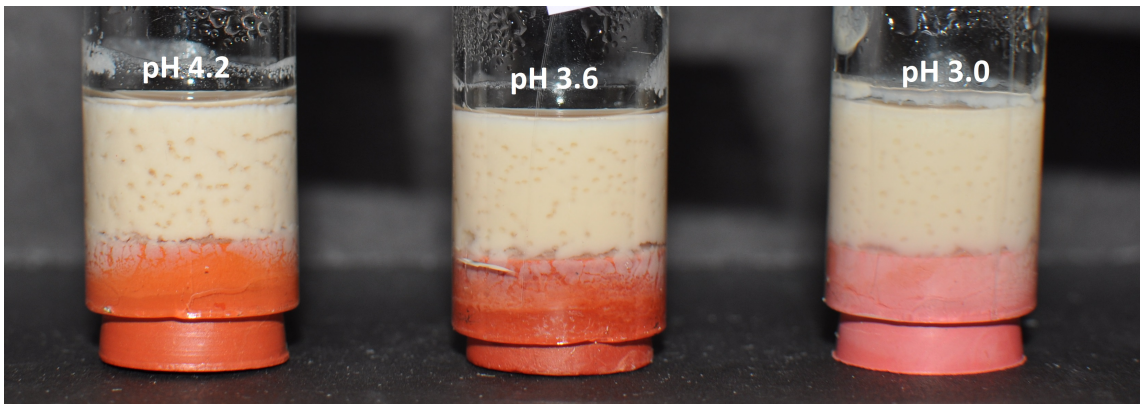


Figure A.2: Pea protein gels (150mg PPI/mL water) at different pH (4.2, 3.6 and 3.0)

A.2 Granule ghost

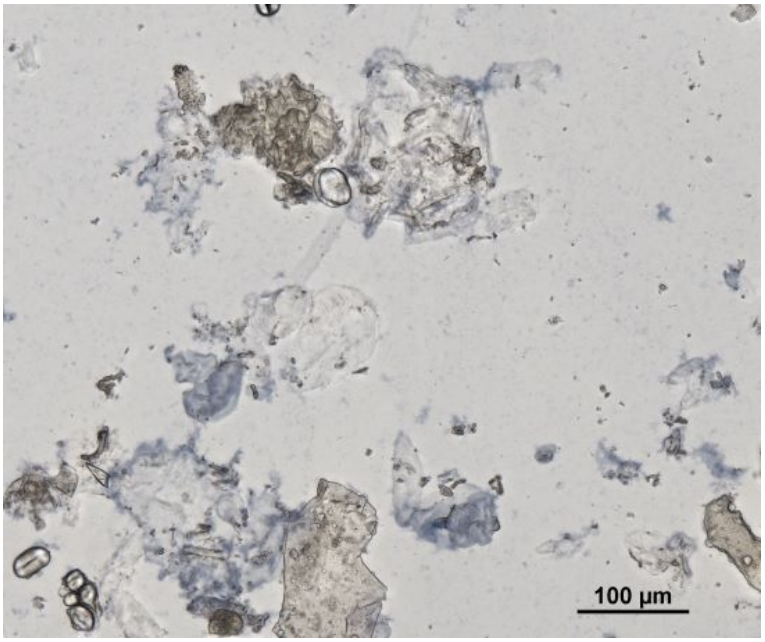


Figure A.3: Light micrograph of heat treated (95°C, 30 min) insoluble-fraction indicating presence of granule ghosts. Staining: Iodine, Objective: 10X

A.3 Dynamic rheology (heating)

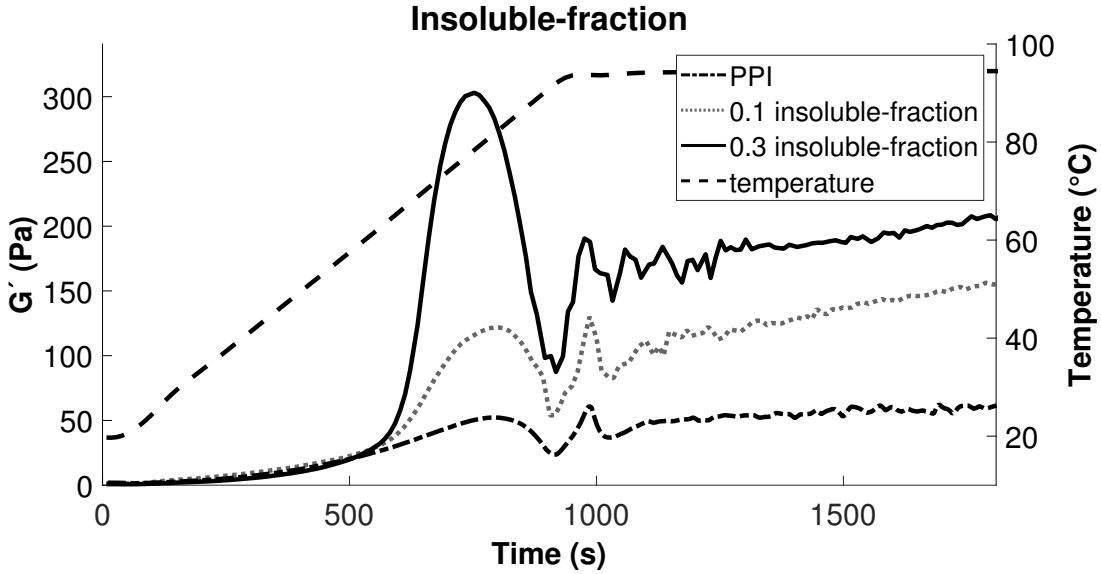


Figure A.4: G′ as a function of time during temperature change simulating the gelation process of pea protein gels (150 mg/mL) with different ratio (insoluble-fraction/protein) of insoluble-fraction added. Zoomed in on the heating part of the temperature ramp.

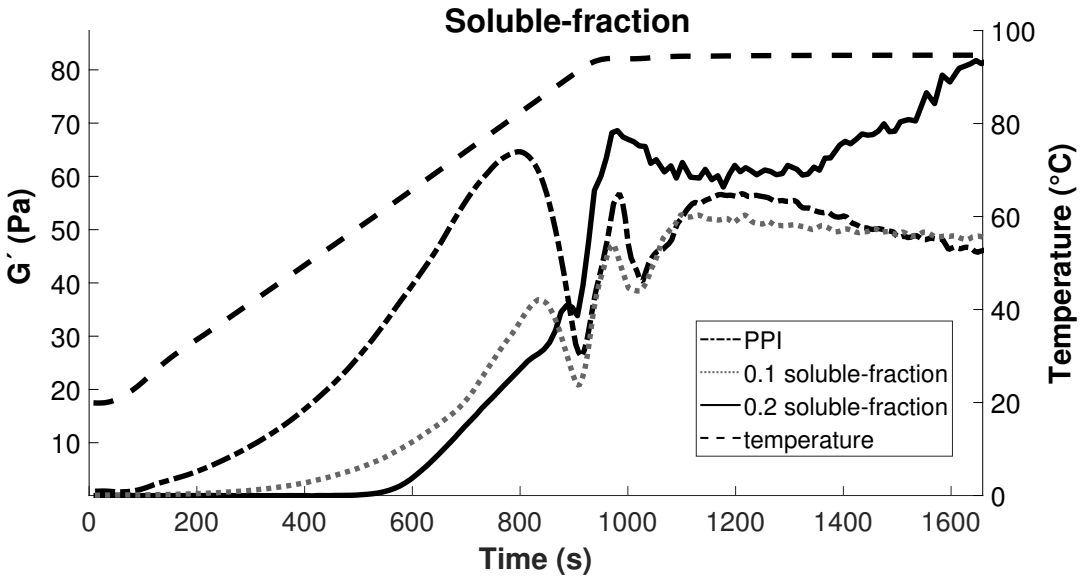
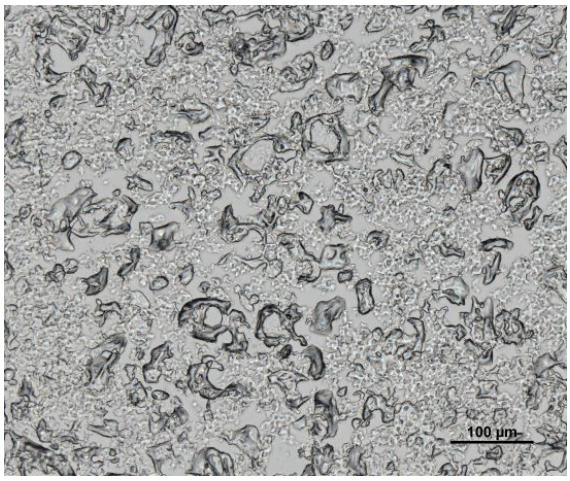
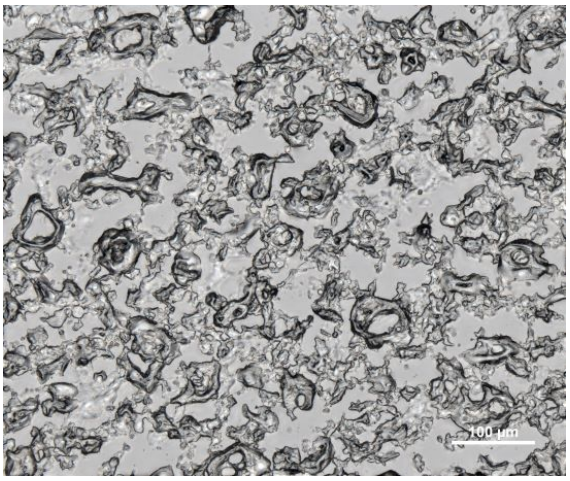


Figure A.5: G′ as a function of time during temperature change simulating the gelation process of pea protein gels (150 mg/mL) with different ratio (soluble-fraction/protein) of soluble-fraction added. Zoomed in on the heating part of the temperature ramp.

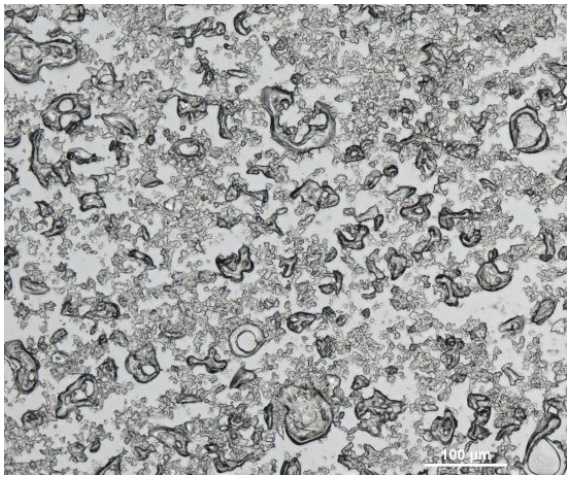
A.4 Light microscopy (unstained)



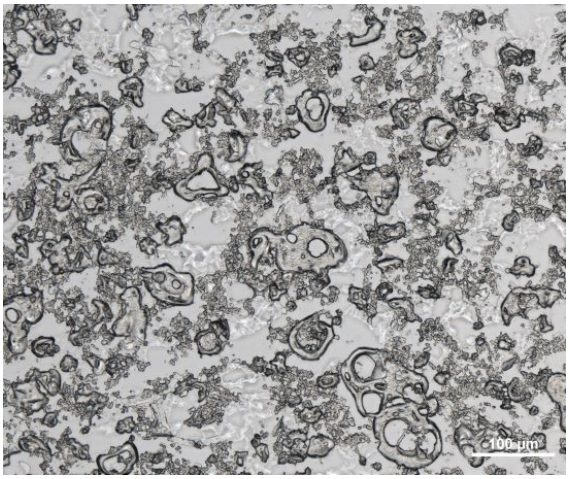
(a) pH 3.0; PPI, 10X



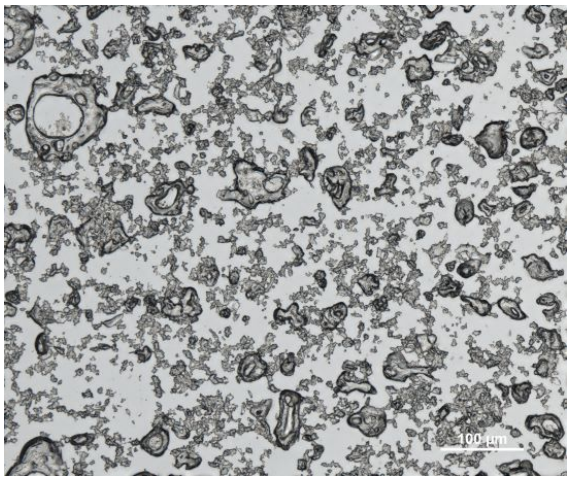
(b) pH 3.0; 0.3 insoluble-fraction, 10X



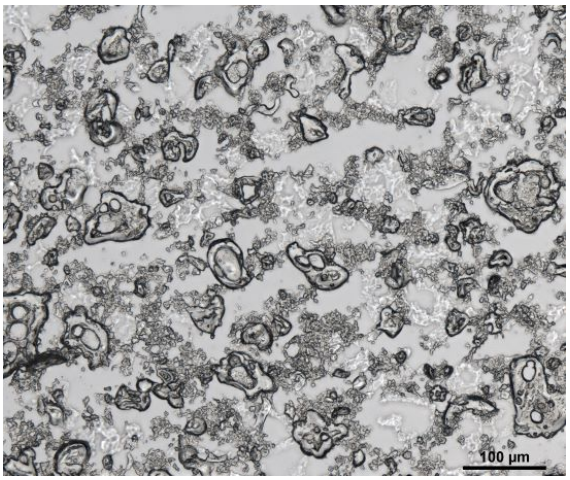
(c) pH 3.6; PPI, 10X



(d) pH 3.6; 0.3 insoluble-fraction, 10X

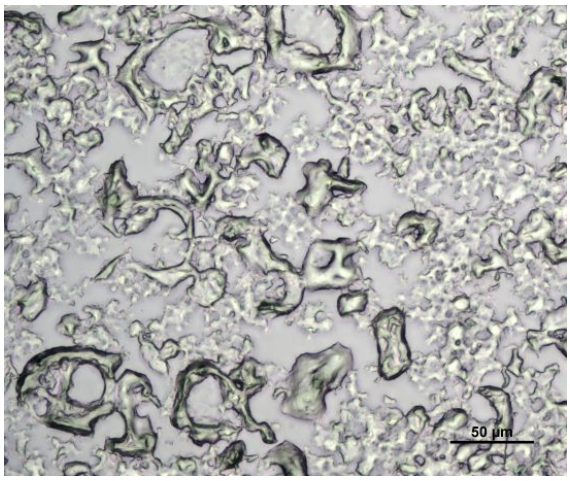


(e) pH 4.2; PPI, 10X

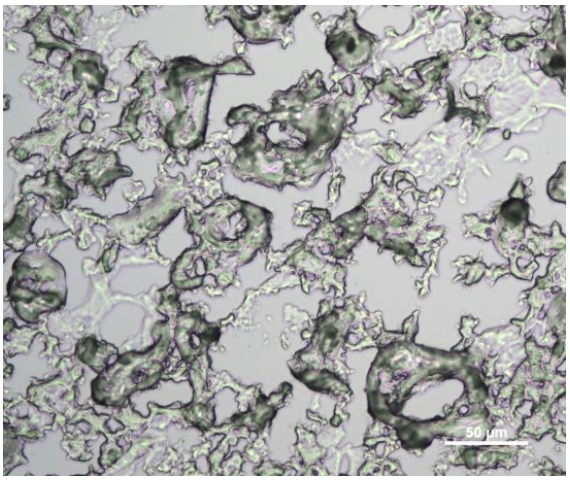


(f) pH 4.2; 0.3 insoluble-fraction, 10X

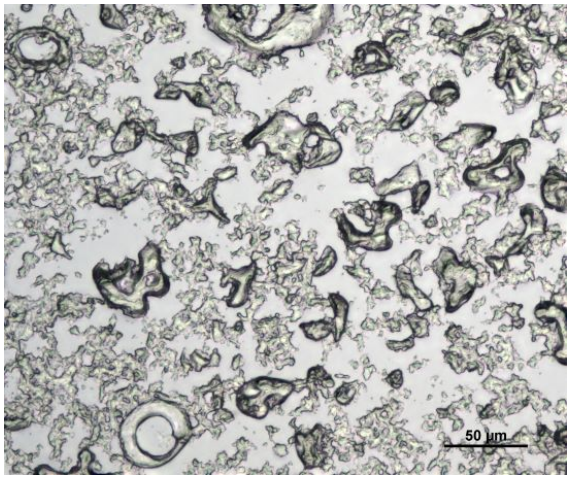
Figure A.6: Light micrographs of pea protein gels (150 mg PPI/mL water) at different pH and with or without insoluble-fraction added to a ratio of 3:10 (insoluble-fraction:PPI). Scale bars correspond to 100 µm. Objective/magnification: 10X. Staining: none.



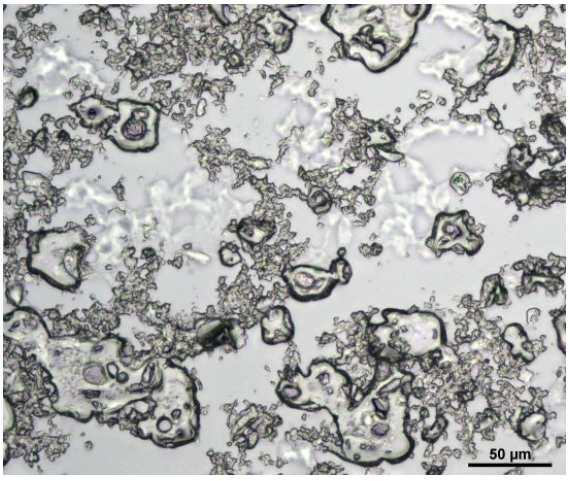
(a) pH 3.0; PPI, 20X



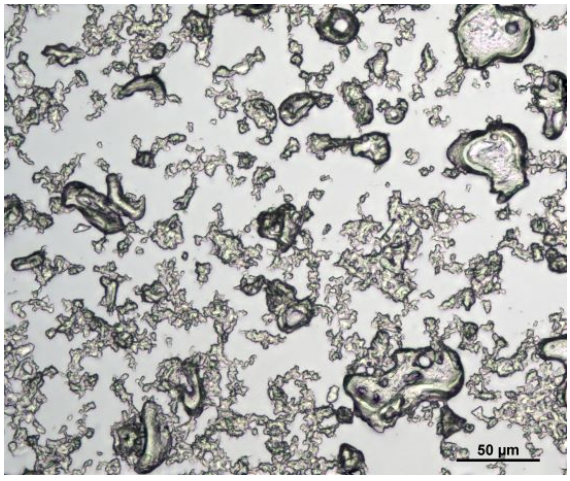
(b) pH 3.0; 0.3 insoluble-fraction, 20X



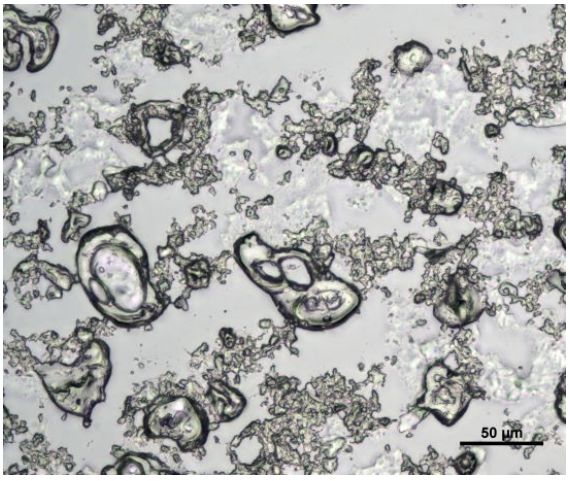
(c) pH 3.6; PPI, 20X



(d) pH 3.6; 0.3 insoluble-fraction, 20X

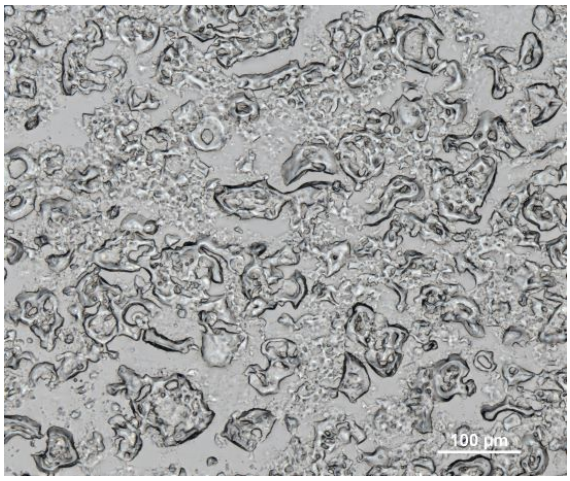


(e) pH 4.2; PPI, 20X

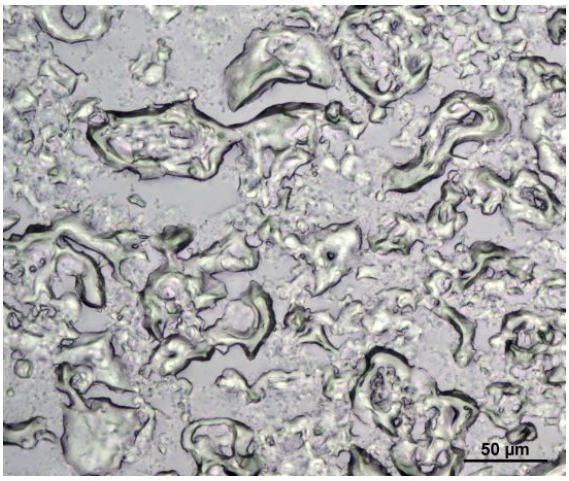


(f) pH 4.2; 0.3 insoluble-fraction, 20X

Figure A.7: Light micrographs of pea protein gels (150 mg PPI/mL water) at different pH and with or without insoluble-fraction added to a ratio of 3:10 (insoluble-fraction:PPI). Scale bars correspond to 50 µm. Objective/magnification: 20X. Staining: none.



(b) pH 3.0; 0.1 soluble-fraction, 10X



(b) pH 3.0; 0.1 soluble-fraction, 20X

Figure A.8: Light micrographs of pea protein gels (150 mg PPI/mL water) with soluble -fraction added to a ratio of 1:10 (soluble-fraction:PPI). Scale bars correspond to 50 µm. Objective/magnification: 10X/20X. Staining: none.

A.5 CLSM

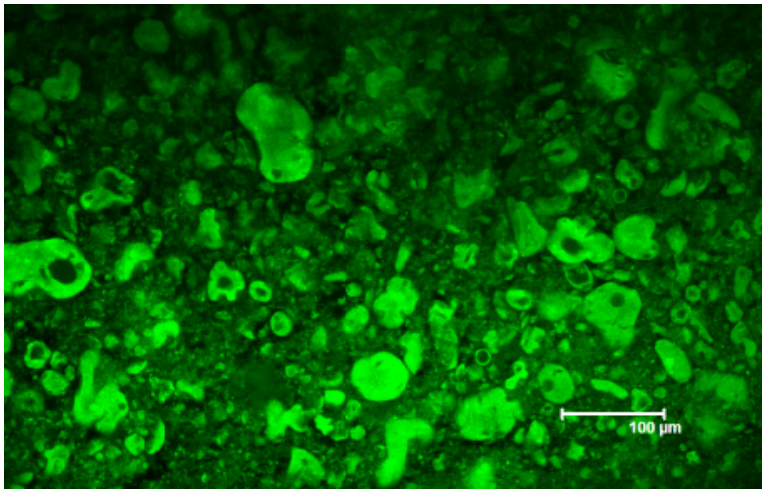


Figure A.9: Micrograph obtained using CLSM of PPI (150 mg PPI/mL water) gel stained with rhodamine B. Scale bar: 100 µm

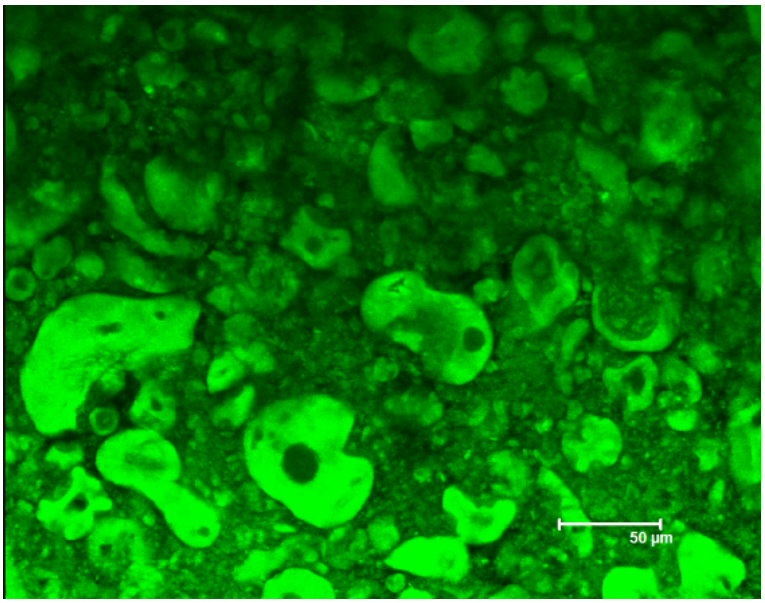


Figure A.10: Micrograph obtained using CLSM of PPI (150 mg PPI/mL water) gel stained with rhodamine B. Scale bar: 50 µm

A.6 Moisture loss

Sample	pH	Moisture loss	Number of replicates
PPI	3	8.98±1.44*	6
0.1 insoluble-fraction	3	9.90 ± 0.72	4
0.3 insoluble-fraction	3	7.19 ± 0.78	2
0.3 insoluble-fraction	3.6	14.4 ± 0.70	2
0.3 insoluble-fraction	4.2	22.77 ± 0.35*	2

Table A.1: Moisture loss calculated according to equation 2.10 for pea protein gels (PPI) and pea protein/insoluble lentil fraction gels with diffrent ratios of insoluble fibre (0.1 and 0.3 gram insoluble fibre/gram PPI). *Gels did not fully retain their structure during centrifuging.