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Faculty of Natural Resources and
Agricultural Sciences

Gelation of faba bean protein isolates

-effect of ionic strength, pH and extraction
procedure

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Gelation of faba bean protein isolates: effect of ionic strength, pH and extraction procedure

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Abstract

The livestock sector plays a major role in the climate change that has occurred since the industrial revolution and which is now the most acute challenge humans are facing. Consequently, a sustainable source for proteins are warranted and legumes are set to lead the way. In Sweden, fava beans have the potential to replace both animal proteins and imported soy beans, but it is still to reach consumers. The aim of this thesis was to study gelation properties of two different protein isolates from fava beans. One isolate was extracted by solubilizing proteins at alkaline pH and then precipitated at the isoelectric point (alkaline extraction). The other method was partly based on the procedure during tofu production (bean milk extraction). Both isolates were freeze-dried prior to use. Effects of protein concentration, pH and NaCl content on gelation properties and gel characteristics were analysed by compression tests and light microscopy. Results displayed that the least gelling concentration was lower at pH 5 compared to pH 7 and that the addition of 2% NaCl lowered it even further. However, lowering pH and increasing NaCl content both weakened the gels at a given concentration of 15% protein (w/w). Gels made from alkaline extracted proteins made the hardest and stiffest gels at pH 7, meanwhile gels made from the bean milk isolate were harder, stiffer and seemed to have better cohesive properties at pH 5. These finding gives the possibility to use different isolates and adjusting pH and NaCl content in different foods to obtain the desired texture.

Keywords: fava bean, gel, protein, NaCl, compression, food

Sammanfattning

Djurhållningssektorn har haft en stor påverkan på klimatförändringen som pågått sedan den industriella revolutionen och är den mest akuta utmaningen som mänskligheten står inför. Därför bör vi eftersträva att finna en hållbar proteinkälla och baljväxter kan vara svaret. I Sverige har åkerbönan potential att ersätta både animaliskt protein och importerad soja, men det har ännu inte nått konsumenter. Målet med detta arbete var att studera två proteinextrakt från åkerbönan och dess gelningsegenskaper. Ett av isolaten extraherades genom att lösa upp proteiner vid basiskt pH och för att sedan fälla ut det vid den iso-elektriska punkten. Den andra extraktionsmetoden baserades på en process liknande den som används vid tofuproduktion. Både isolaten frystorkades innan användning. Effekten av pH, proteinkoncentration och NaCl-innehåll på gelningsegenskaperna utvärderades genom kompressionstester och ljusmikroskopi. Resultaten tyder på att den kritiska gränsen för gelning är lägre vid pH 5 och tillsatt 2% NaCl (w/w) jämfört med vid pH 7 och utan tillsatt salt. Både sänkt pH och tillsatt salt resulterade däremot i svagare geler vid en given proteinkoncentration (15% w/w) förutom i geler från bönmjölkeextraktet där justering till pH 5 resulterade i fortsatt hårda geler. Proteiner från alkaliextraktet gav generellt hårdare geler, speciellt vid pH 7. Vid pH 5 däremot, resulterade bönmjölkeextraktet i hårdare geler och visade bättre kohesionsegenskaper. Resultaten från denna studie tyder på att det finns en bra möjlighet för att använda proteinisolat från åkerbönan i livsmedelsindustrin där man vid produktion kan justera pH, saltkoncentration eller använda olika isolat för att uppnå den önskade texturen.

Nyckelord: Åkerböna, protein, gel, kompression, NaCl, livsmedel

Preface

This thesis was written to fulfil the requirements for a Master's degree in Food Science from the Swedish University of Agricultural Sciences. The thesis was a result from my interest in finding a sustainable protein source that have the potential to decrease the impact of food production on the environment. Humanity is facing difficult challenges and unless we, especially in western countries, change our habits, the consequences will be disastrous.

My supervisors during this work have been Daniel Johansson at the Swedish University of Agricultural Sciences and Xinmei Feng at RISE. I would like to thank Daniel Johansson for the patience and continuous support and for helping me throughout the whole research. I have learned a lot from your inputs and ideas, which have been vital for the outcome of this research. Also, I would like to thank Xinmei Feng for helping in designing the method, Monika Johansson for sharing thoughts and for also supervising me during my bachelor's thesis. Special thanks to Siavash Ehsanzamir, my brother and structural engineer at Ramböll for helping me interpret results.

I hope that results from this study can be used in food production and in further studies on fava beans as a future protein source.

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1 Introduction

One of the most acute and major challenges humanity is facing is climate change and the evidences are compelling. The average surface temperature of our planet has risen with 1.1°C since the late 19th century, causing decreased snow cover, desertification and heightened sea levels. Some of the heat is also absorbed by oceans, leading to shrinking ice sheets, glacial retreat, declining arctic sea ice and ocean acidification. The current warming trend is of particular significance since most of it is extremely likely to be the result of human activity since the industrial revolution (NASA, 2016). There is no question that increased level of greenhouse gases must cause the earth to warm in response. The heat trapping property of carbon dioxide and other gases due to their ability to transfer infrared energy through the atmosphere have already been demonstrated (Kushnir, 2000).

The livestock sector plays a major part and is responsible for almost one fifth of all greenhouse gases. It also accounts for one third of the planet's used land surface (Steinfeld *et al.*, 2006). Without diminishing issues concerning animal welfare and environmental impacts, it is vital to recognise the importance of the livestock sector both socially and politically. It accounts for 40% of agricultural gross domestic products (GDP). It employs 1.3 billion people and creates livelihoods for one billion of the world's poor (Steinfeld *et al.*, 2006). However, progression in this rate is not sustainable. An effectivization of the sector is warranted and in western countries most possibly a decrease of its production. Despite all warnings, the global production of meat was projected to more than double from 229 million tonnes in 1999/01 to 465 million tonnes in 2050. At the same time, the environmental impact per unit of livestock must be cut by half just to avoid increasing the level of damage beyond its present level (Steinfeld *et al.*, 2006).

Plant based proteins and locally adapted crops are two approaches to decrease the ramifications of livestock production. One deal with the problem directly by decreasing the demand for animal based proteins, meanwhile the other decreases the consequences of importing animal feed from e.g. the Amazonas area. Cutting forests and ploughing forests in order to sow soybeans frees carbon and increase the levels

of carbon dioxide in the atmosphere consequently contributing to the current climate change. The production systems that are used in Brazil and many other exporting countries of soybeans uses extensive amounts of fertilizers that requires energy to produce. Adding on all the feed that needs to be transported over great distances. In 2008, Sweden imported more than 350 000 tonnes of soybeans and 90% of it were used as animal feed (Heimer, 2010).

Soybeans belongs to the legume family, which are protein rich plants, grown agriculturally, primarily for their grain seed that is used as food and animal feed. Other well-known and widely used legumes are chick peas, lupins and fava beans.

1.1 Aim

The aim of this thesis is to study the gelation properties of proteins from the *Vicia Faba* variety; Gloria. The effect of changing various parameters such as protein concentration, pH and NaCl content are analysed with light microscopy and compression tests. The objective is to present results, which can be used in food production and in further studies on fava beans. This study is limited to the resulting gels and their texture properties.

1.2 *Vicia Faba*

Vicia Faba, also known as fava bean, faba bean or broad bean is a major food and feed legume. It is believed to originate from the Mediterranean region or southwestern Asia where it is a crucial source of proteins in food (Tanno & Willcox, 2006). The world production of fava beans were 4.3 million tonnes in 2010 compared to soybean's 262 million and pea's 10 million tonnes (Fouad *et al.*, 2013; Sharma *et al.*, 2013). Fava beans are cultivated in more than 50 countries with China as its largest producer (Geissler, 2009). In the northern hemisphere it is sown during the spring and in warmer areas, during winter as it needs cooler temperatures for best development.

Due to its high protein content, it is used today as both human food and feed for cattle. Most varieties contain tannins thus making it unsuitable as feed for monogastric animals and must be pre- heated before human consumption. White flowering fava beans, such as Gloria, are also available. They are cultivars with zero to minimal levels of tannins. Generally, cultivars containing tannins (colour- flowering) grows taller, generate higher yield, have better weed resistance but later maturing. White- flowering mature earlier but are more susceptible to diseases and pests since tannings are compounds used by crops as a defence mechanism against pathogens. The compound decreases the *in vivo* degradation of proteins in monogastric animals

leading to protein deficiency. Tannins have also been reported to affect the taste of feed negatively (Multari *et al.*, 2015; Crépon *et al.*, 2010).

Vicia Faba is also used with very good results as a break crop for cereals in agricultural systems. Studies have shown that intercrops between them increased the yield for both plants. Due to their tolerance for each other's pests, *Vicia faba* decreases the occurrence of cereal cyst nematodes (CCN), which leads to increased yield. The crop also has the ability to fixate atmospheric nitrogen in symbiosis with rhizobium bacteria, limiting the need for additional nitrogen through the manure for itself and the subsequent plant (Crépon *et al.*, 2010). It is in many regards a good plant with good yield, but its harvest can be decreased by fungi such as *Peronospora viciae*, *Botrytis faabae* (chocolate spot) and *Aschochyta fabae* (ascochyta blight). In recent years *Phytophthora pisi* have also become more common (Raynes, 1994). Consequently, it is crucial that the crop is not grown in the same field more than 6- 7 years apart and not less than 500 meters between fields to avoid spread by the wind (Jordbruksverket, 2014). Sowing time should also be considered to decrease risks of infection. In Sweden, the best sowing time is between Mars and April. Earlier sowing can promote disease development (ScandinavianSeed, 2017). Delayed sowing can reduce yield due to drought stress as the temperature is increased at flowering. Fava beans take about 110- 130 days to mature, depending on the variety.

The crop is sensitive to drought and tolerates water logging better than most legumes. It is therefore best grown in loams due to its high water holding capacity and often nutritional soil (Jordbruksverket, 2014).

1.2.1 *Vicia Faba* in Sweden

Fortunately, loams are the dominating soil type in Sweden's farmland, which means that domestic production of fava beans have the potential to replace imported soybean. The climate is also well fitted for the fava bean since it thrives in cool temperatures, are frost tolerant and do not do well in hot and dry climates.

In 2016, 103 900 tonnes were harvested and half of it was grown in the production area between Gothenburg and Stockholm known as "Götalands norra slättbygder (Gns). Last year's harvest was an increase by 52%, compared to the average of the last five years (Jordbruksverket, 2017). The sudden intensification in recent years may be a result of the increased interest in domestic protein feed. It may also be a response to the new type of financial support, which was introduced in 2015 with the aim to decrease the European agriculture's impact on the climate. Increased harvesting of domestic legume generates an agriculture less dependent on imported feed.

1.2.2 Protein content in *Vicia faba*

Commercial fava bean grain has a protein content of 24- 30%, depending on the variety. Just like other legumes, fava beans accumulate a lot of proteins during seed development. The major storage proteins are globulins, which consists of two high-molecular- weight proteins called legumin and vicilin, or 11S and 7S respectively. Globulins are soluble in salt solutions and dissociate into their subunits by exposure to extreme pH values. They comprise about 69- 78% of the total seed proteins. Globulins are generally rich in aspartic acid, glutamic acid, leucine and arginine. In the developing seed, vicilin is formed before legumin but the latter is synthesized at a faster rate and in the mature seed it predominates. Quantitatively legumin comprises about 40- 45% of the protein fraction and vicilin about 20- 25%. Followed by legumins and vicilins; albumins, prolamins and glutelins are the major protein portions in fava beans (Multari *et al.*, 2015; Makri *et al.*, 2006).

1.2.3 Nutritional properties

The amino acid composition of the protein in fava beans are quite similar to other legumes and are characterized by a generally good nutritional quality with the exception of low sulphur amino acid and tryptophan concentration. In a diet it is easily compensated for by eating it with grains. In table 1, the amino acid composition of legumin and vicilin, which are the dominating protein fractions in fava beans, are shown.

Table 1. *Amino acid composition of legumin (11S) and vicilin (7S) fractions in fava beans. Values are amino acid residues shown as a percentage of the total number of residues present (Multari et al., 2015)*

Amino acids	V. faba 11S (%)	V. faba 7S (%)
Aspartic	10.60	11.60
Threonine	4.28	3.27
Serine	6.50	6.59
Glutamic	16.40	15.30
Glycine	7.40	5.00
Alanine	6.10	4.87
Valine	4.91	4.90
Cystine	0.80	0.31
Methionine	0.59	0.31
Iso- leucine	3.98	5.12
Leucine	7.84	9.21
Tyrosine	2.61	2.59
Phenylalanine	3.56	5.20
Lysine	4.57	7.13
Histidine	2.44	1.95
Arginine	7.95	5.59

Along with proteins, carbohydrates are the largest group making up to 40- 50%. Like most legumes it has a low content of fat, around 2%. Crude fibre content can vary from 8- 15%, All estimation differs greatly between varieties. Fava beans also holds non- nutrient secondary metabolites shown to be beneficial to human health.

It is a staple food in Egypt where it is an important ingredient in the dish “ful medames”. Although it is consumed widely in Mediterranean countries, it has yet not reached consumes in markets where meat is the predominant source of proteins in the diet. In such markets it is mainly used in salads (Multari *et al.*, 2015).

Fava beans also contains antinutritional compounds such as phytic acid, saponins, lectins and alkaloids, which in its raw state can reduce digestibility of proteins, leading to pathological conditions. But many of these can be reduced or eliminated by normal food processing methods that occurs during cooking, such as soaking and heat- treatment (Multari *et al.*, 2015; Vidal-Valverde *et al.*, 1998). As mentioned earlier, there are white- flowering varieties with barely non to zero- levels of some of the antinutritional compounds.

1.2.4 Fava beans in food product development

Several studies on substituting parts of a food ingredient with fava bean flour or isolates have been conducted. Gimenez and others (2012) substituted wheat flour with fava bean flour in order to make fortified pasta. The fortified flour had higher water absorption and lower dough developing time. Another study done by the same researcher concluded that the addition of 30% fava bean flour to corn pasta contributed to an increase in the nutritional quality of the pasta without affecting their texture or flavour (Giménez *et al.*, 2016). Further studies made on the characteristics of extruded fava beans maintained that protein and starch content was not affected in the extrusion process of fava beans, hence could be used in extrusion cooking with wheat (Liene & Sandra, 2016). Studies on the addition of fava bean flour to wheat flour to make pita bread has also been conducted. Sensory analysis showed that pita bread produced from flours replaced with 5% and 10% fava bean flour did not affect sensory acceptability whereas all breads were improved nutritionally (Ajo, 2013).

1.3 Gelation in foods

Many food products contain components with the ability to form gels during heating or cooling. This property is vital in many foods such as yogurts, eggs, jellies, meat and products made from them. The gelling agents in foods are usually proteins or polysaccharides. There is no clear definition of a gel, but they are commonly known as three- dimensional networks, with the ability to entrap a large amount of water by aggregated biopolymers or colloidal particles. The result is a structure with the characteristics of a solid material. It can also be considered as a three- dimensional network with high moisture content that resists flow during pressure. They are held together by weak intermolecular bonds like hydrogen bond, electrostatic forces, Van der Waal forces and hydrophobic interactions (Fleming *et al.*, 1975). Some gels with proteins as the gelling agent are held by disulphide bonds between cysteine residues.

The gel formation can either be triggered by heating or cooling. Example of those are egg white and gelatine, respectively. The gelation process largely depends on temperature, concentration of gelling agents, pH, presence of ions and time. The limit to how low the concentration of a gelling agent can be is defined as the least gelling concentration (LGC) or the critical concentration, and it differs for every protein/ polysaccharide. (Hartmann & Schmandke, 1988).

1.3.1 Gelation of fava beans

Previous studies made on gelling properties of fava bean flour indicated a firm gel in low flour concentration (12% at pH 7) and an overall good gelling capacity over a wide range of pH. One study obtained a least gelling concentration of 14% for protein isolates (Fernández-Quintela *et al.*, 1997). However, since gels have an incomplete definition, the results vary greatly between studies. Other differences are extraction methods and used conversion factors when determining protein content. Besides protein content, different methods also provide varying amount of starch granules, which may have a significant effect on gel strength. In food stuff e.g., surimi- based products; starch is added to enhance the gel strength (Hunt *et al.*, 2009). During gel formation, starch granules absorb water and swell. When heated, they interact with protein and gelatinize, thus increasing gel strength (Paker & Matak, 2017). Studies have shown that the effect starch granules have on protein gels is also dependent upon the starch type because of their differences in amylose to amylopectin ratio and swelling power. Potato starch for example, has a higher content of amylopectin than wheat starch and therefore produces firmer and more cohesive gels (Kim & Lee, 1987). The effect starch granules from fava beans have on protein gels is relatively unknown and will not be considered in this study but may still affect the results.

The gelation process from proteins in fava beans is thermo- irreversible. Sufficient heat causes protein denaturation, where after disulphide bonds are formed and hydrophobic amino acid residues are exposed. Further heating causes aggregation and interactions between proteins, forming a gel. pH primarily affects the net charge of the proteins in the solution. At the isoelectric point, due to a net charge of zero, protein aggregation is favoured rather than protein denaturation, which results in a texture reminding of a curd instead of a gel (Makri *et al.*, 2006). Vicilin in fava beans have an isoelectric point of 5.5, meanwhile legumin has an isoelectric point of 5.0 (Danielsson, 1950).

As mentioned earlier; globulins are soluble in salt solutions. At alkaline pH, increase in ionic strength e.g. by the addition of NaCl lowers the solubility of proteins and will have the same effect as adjusting the pH closer to the isoelectric point. Chen and others observed increasing heterogeneous gels from soybean proteins when increasing NaCl content (Chen *et al.*, 2017a; Chen *et al.*, 2017b).

1.4 Texture analysis- compression test

There are various tests to analyse mechanical properties of materials such as tensile tests, penetration tests and in this case; compression tests. It is one of the most common texture tests used and it is important for the understanding of behaviour of food materials during production and consumption. A sample is placed on a flat surface and compressed, commonly by a cylindrical shaped form. Tests are performed by compression to a given force (stress), a given position or a percentage of the original height of the sample, also known as strain. After compression a stress-strain curve is obtained for further analysis. Stress is the force applied per unit of area and reflects the firmness of the gel (y- axis). The hardness of a material is defined as the maximum stress at the first break. Strain (x- axis) is an indication of cohesive properties. Cohesiveness may be measured as the rate at which the material disintegrates under mechanical action. Fracturability or brittleness is defined as the strain and stress at the first significant break in the curve. Adhesiveness is a difficult parameter to estimate in compression tests of gels. It is defined as the negative force area as the probe is moving back. The difficulties lie in assuring that gels do not stick to the probe after compression since the added weight to the probe will give a false curve. Stress- strain curves are affected by sample- width and height and the ratio between them should consequently be equal to be comparable. Generally, the sample should be cylindrical shaped with a length to diameter ratio of >1 . The results can also be affected by the used compression force and speed.

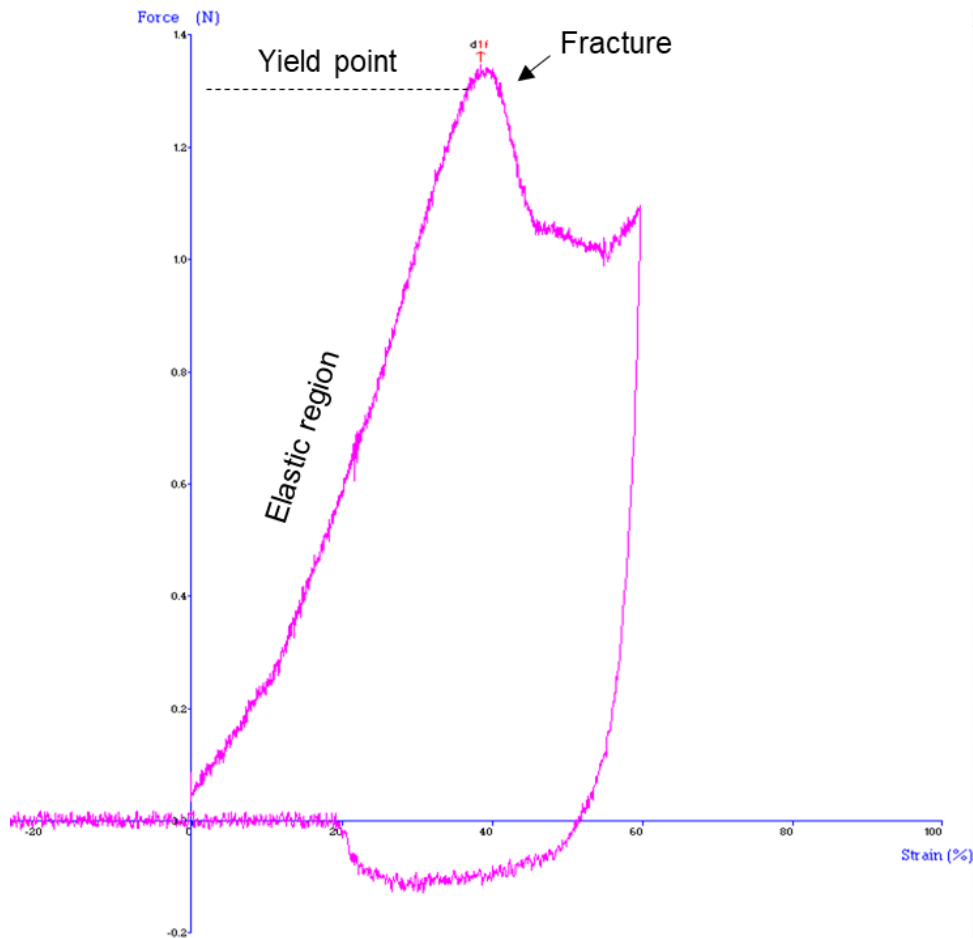


Figure 1. Example of a stress- strain curve obtained after compression test.

Figure 1 is a typical stress- strain curve obtained from a compression test. Even though stress is in reality; force divided by compression area, the ratio between force and stress are equal when the compression area is the same. One can initially see a linear relationship between stress (force) and strain. This is the elastic region of the curve and a deformation of the material within this region would only return to its original shape. The relationship is defined as Hooke's Law, where the ratio between stress to strain is constant. The ratio between stress and strain in this region is called Young's modulus and curves with high values are stiff meanwhile those with low are flexible. The minimum amount of stress that is required to cause a permanent deformation is called yield point. From this point and forward, the material is in the plastic region. If the curve has a sudden decrease as in figure 1, the material is seen as brittle. If it is declining slowly it is a ductile material (Roynance, 2001). The ductility of a material is a measurement of the extent to which a material will deform

before fracture. Ductility is an important factor in many forming operations in food e.g. extrusion (TextureTechnologies, 2014).

2 Materials and methods

This thesis focused on gelation of extracted proteins from dehulled fava beans of the variety Gloria. Two different extraction methods were used and gelation as a function of pH, concentration and sodium chloride concentration was studied. Means and standard deviation was calculated by using the software Minitab.

Table 2. *Chemical constituents in the fava bean variety; Gloria (Jezierny et al., 2010; Makkar et al., 1997)*

Variety	Crude protein	Ash	Starch	Lipid content	Crude fibre
Gloria	30%	4%	42%	2%	13%

2.1 Protein extraction

Protein extraction from fava beans were carried out with two different methods. One is partly based on the method used for tofu production; soaking, pH adjustment and removal of larger components. The other method is based on alkali extraction of protein from milled beans. Extracts from both methods were later freeze dried for further handling.

2.1.1 Bean milk extraction

Fava beans were soaked for 10 h with a water to bean ratio of 2:1. Subsequently, more water was added to obtain a water to bean ratio of 3:1. The beans were later mixed in a high- speed blender (2 x 2 min). pH was adjusted to 8 with 5 M NaOH and the slurry was stirred for 1 h before centrifugation at 3700 g (20 °C, 15 min). The supernatant was saved and freeze- dried for 4 days. The extraction was performed two times but only one batch was used in protein and dry content analysis.

2.1.2 Alkaline extraction

Finely milled fava bean flour was mixed with distilled water at a solid-to- solvent ratio of 1:10 (w/v) to make a slurry. The pH of the slurry was adjusted to 8.0 with 5 M NaOH and incubated at room temperature (20 ± 2 °C for 1 h under continuous stirring). The slurry was then centrifuged at 3700 g (20 °C, 30 min) in a Multifuge 3 S/ 3 S-R centrifuge to remove insoluble materials. The pellet was discarded, and the pH of the supernatant readjusted to 4.0 with 6 M HCl. The supernatant was incubated at room temperature for 2 h under continuous stirring and later centrifuged at 3700 g (20 °C, 30 min). The pellet was collected and re- dispersed in distilled water in a ratio of 1:10 (w/v) with adjustment of pH to 4.0 if required. The re- dispersed suspension was centrifuged at 3700 g (20 °C, 30 min), following this, the pellet was collected, freeze-dried and grounded.

2.1.3 Protein determination and dry content

Protein content of extracted isolates were determined by the Kjeldahl method. It is a method used commonly for estimating protein content in foods by quantitatively determining nitrogen content and converting it to protein content with the conversion factor 6.25. Protein content for this study was calculated by using the conversion factor 5.4 instead of 6.25, which have been reported to be more accurate for legumes.

For dry content analysis, protein extracts were heated for 4 h in 105 °C. Samples were weighed before and after heat- treatment to calculate dry content.

2.2 Gel formation

Gel formation properties of the extracted proteins was examined according to figure 2. Every point was replicated three times with two measurements at each replicate. Gels from the alkali extracts were made from three different batches, meanwhile gels from the bean milk extracts were made from a total of two batches. Gels were studied with compression tests and microscopy.

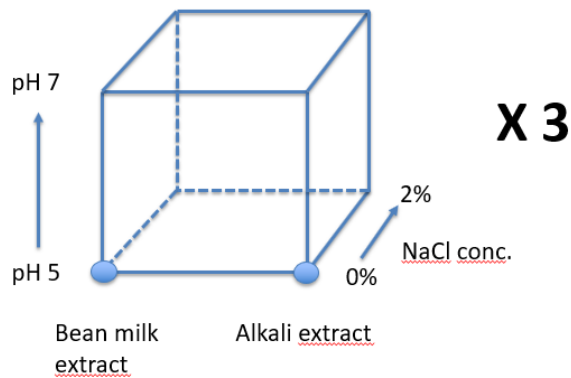


Figure 2. Gels were made according to the picture where every parameter was performed three times. (Sohail Ehsanzamir, SLU)

2.2.1 Gelation

Protein isolates were dispersed in distilled water to obtain a final concentration of 15% (w/w) pure protein-to-solvent ratio, after pH adjustment. The pH of the alkali extracts was adjusted to 8.0 with 5 M NaOH during continuous stirring and thereafter stirred for 1 h to solubilize as much proteins as possible. The slurry was then adjusted to 7.0 or 5.0 with 6 M HCl. After each adjustment, the slurry was stirred for 15 minutes. Bean milk isolates already had a pH of 8.0 and therefore stirred for only 15 minutes and then directly treated with HCl. Gels were made in beakers that were sealed with aluminium folia and rubber bands. 2% (w/w) NaCl were added to samples. The beakers were put in 95 °C water bath (30 min) and cooled to room temperature (20 °C), also in a water bath.

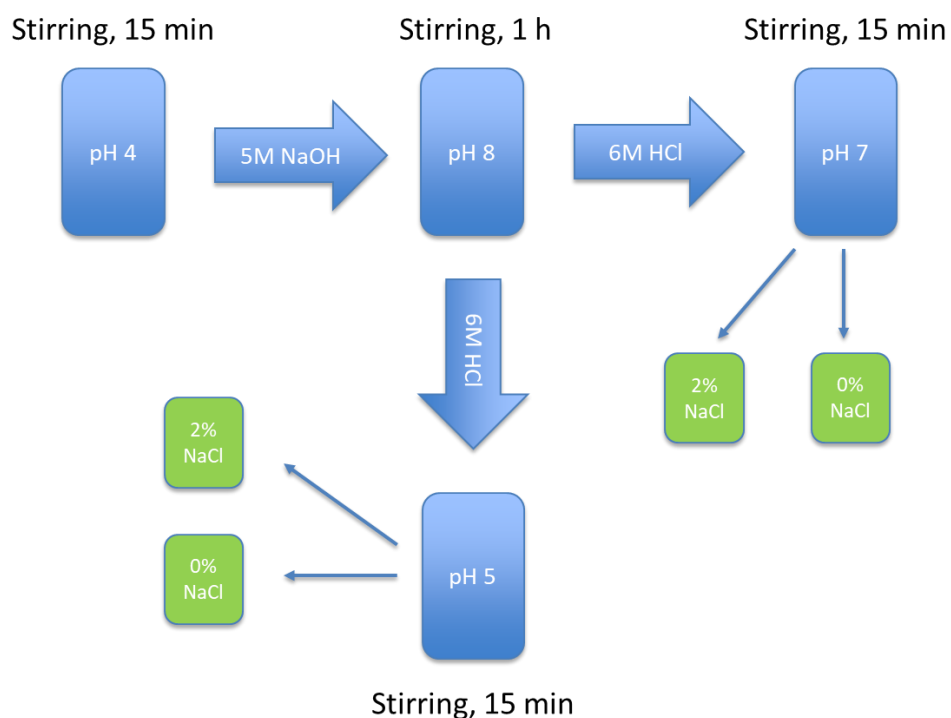


Figure 3. Procedure for making gels from alkali extracts and bean milk extracts. Alkali extracts starts at pH 4 meanwhile bean milk extracts at pH 8, which were also stirred for 15 min instead of 1 h (Sohail Ehsanzamir, SLU).

2.2.2 Least gelling concentration

Gels with protein concentrations between 6- 15 % were made at pH 5 and pH 7 in test tubes with the width of 1 cm. Samples containing 2% NaCl were also made. The least gelling concentration was defined as the protein concentration at which there was no observed flow of gels when test tubes were put upside- down. Gels were recognized as firm when able to take them out from the test tubes without falling apart. Samples were divided in no gels (-), soft gels (O) and firm gels (X).

2.2.3 Compression test

Samples were cut in cylindrical shaped forms with a diameter of 11 mm and height of 15 mm. Compression tests were carried out with a Stable Micro Systems TA-XT2. The texture analyser was fitted with a 35 mm in diameter cylindrical metal compression plate. Trigger force was set at 0.98 N. Pre-and post-test speed was adjusted to 2.00 mm/s and test speed to 1.00 mm/s during measurements. The samples were compressed to reach 60% of its height.

2.2.4 Microscopy analysis

Gels were cut in approximately 2 x 2 mm and put in 1 M phosphate buffer (pH 7.2) with a mixture of 4% formaldehyde and 1% glutaraldehyde solution for 24 h. Plastic imbedding was performed with a tissue processor (Leica EM TP) according to the program presented in table 3.

Table 3. Program for plastic imbedding of gels

Steps	Solutions	Time	Temp °C	Agitation
1	Phosphate buffer	10 min	18	Yes
2	Phosphate buffer	10 min	18	Yes
3	30% ethanol	10 min	18	Yes
4	50% ethanol	10 min	18	Yes
5	50% ethanol	10 min	18	Yes
6	70% ethanol	10 min	18	Yes
7	70% ethanol	10 min	18	Yes
8	90% ethanol	10 min	18	Yes
9	90% ethanol	10 min	18	Yes
10	95% ethanol	10 min	18	Yes
11	95% ethanol	10 min	18	Yes
12	100% ethanol	1h	18	Yes
13	100% ethanol	1h	18	Yes
14	1:1 LR white- 100% ethanol	1h	18	Yes
15	LR white	12h	18	Yes
16	LR white	1h	20	No

After the program, gels were transferred to small gelatin capsules filled with LR white resin. The containers were sealed and heated in an oven (24 h, 55 °C). Samples were cut (1000 nm) with an ultramicrotome (Leica EM UC6) and stained with iodide and light green. Micrographs were taken with a Nikon Eclipse Ni- U Upright Microscope, objective x40 with a N.A 0.95 and image resolution 2560 x 1920.

2.3 Statistical analysis

All statistical analysis was performed using Minitab. Means and standard deviations were calculated, and the sample variance was plotted in individual value plots. One-way ANOVA tests was performed at a significance level of 0.05 followed by pair-wise comparisons. A factorial design was used but not in the statistical evaluation.

3 Results

Gels were named after method of extraction, pH and NaCl content. All names and description of them are shown in table 4. The letter before pH defines which isolates were used, the number after pH states the pH of the gel.

Table 4. *Name and description of tested gels*

Name	Description
APH7	pH 7 gels from alkali extract
APH7 + NaCl	pH 7 gels from alkali extract with NaCl
APH5	pH 5 gels from alkali extract
APH5 + NaCl	pH 5 gels from alkali extract with NaCl
BMPH7	pH 7 gels from bean milk extract
BMPH7 + NaCl	pH 7 gels from bean milk extract with NaCl
BMPH5	pH 5 gels from bean milk extract
BMPH5 + NaCl	pH 5 gels from bean milk extract with NaCl

3.1 Protein content

Protein contents in table 5 are calculated on dry basis. The results show that alkali extracts have higher protein content than bean milk extracts.

Table 5. *Protein content of fava bean flour and different methods of extraction after correction for dry content. Protein content was calculated with the conversion factor 5.4*

Sample	Protein content (%)
Fava bean flour	29.4 ± 0.4
Alkali extract	79.8 ± 3.0
Bean milk extract	61.0 ± 1.4

3.2 Least gelling concentration (LGC)

Lowering pH from 7 to 5 results in a colour change from yellow/brown at pH 7 to white at pH 5. The texture of pH 5 gels reminded more of a curd than a gel. The addition of NaCl in pH 7 also marks a minor colour shift as can be seen in figure 4.

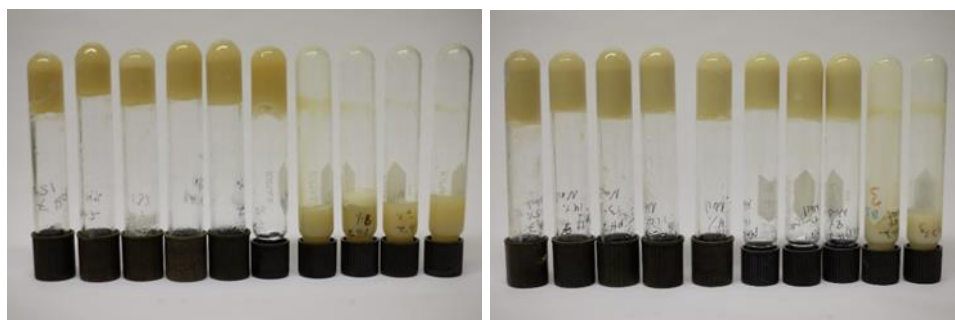


Figure 4. LGC for APH7 (left) and APH7 + NaCl (right). Protein concentration decreases from 15-6% from left to right (Photo: Sohail Ehsanzamir, SLU)

The results from figure 4 and the rest of the samples are compiled in table 6 for an easier overview. Bean milk extracts had an overall lower LGC than alkali extracts. Adjusting pH from 7 to 5 and adding 2% NaCl lowered LGC further in both extracts. The possible effect of NaCl on the LGC of BMPH5 was not observed since it would be out of range. Although LGC varied greatly between samples, they all formed firm gels at 12- 14%.

Table 6. Least gelling concentrations of the different samples where - stands for no gel, O for weak gel and X for firm gel

Concentration %	APH7	APH7 + NaCl	APH5	APH5 + NaCl	BMPH7	BMPH7 + NaCl	BMPH5	BMPH5 + NaCl
6	-	-	-	O	-	O	O	O
7	-	-	O	O	-	O	O	O
8	-	O	O	O	O	O	O	O
9	-	O	O	O	O	O	O	O
10	O	O	O	O	O	O	O	O
11	O	O	O	O	O	O	O	O
12	X	X	O	O	X	O	O	O
13	X	X	O	X	X	X	X	O
14	X	X	X	X	X	X	X	X
15	X	X	X	X	X	X	X	X

3.3 Microscopy analysis

Microscopy analysis of samples stained with iodide and light green are shown in figure 5- 8. APH7 and APH7 + NaCl formed continuous gels, which appeared homogenous in the magnification used and no effect of NaCl was visible in the micrographs (figure 5). When adjusting pH to 5, the continuous gel phase was abruptly and the distance between aggregates were increased (figure 6).

BMPH7 and BMPH7 + NaCl also formed continuous gels but had higher content of starch and other residuals than gels from alkali extracts. In those gels (alkali extracts), the addition of NaCl did not result in any visible difference (figure 7). BMPH5 markedly differed from APH5 by resulting in denser aggregates. NaCl appeared to decrease the density of the aggregates (figure 8).

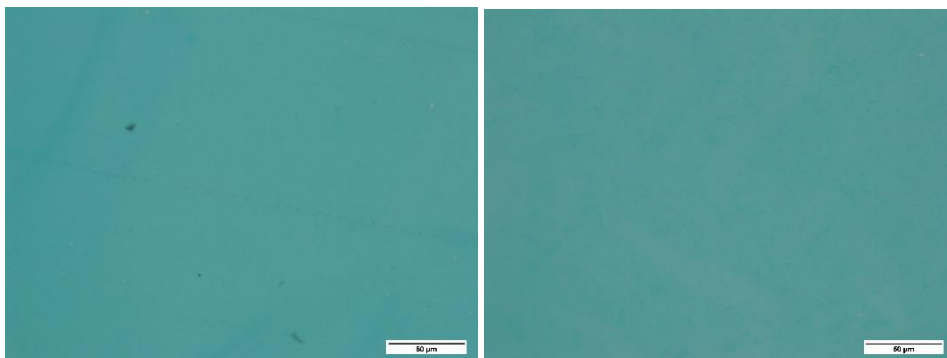


Figure 5. APH7 (left) and APH7 + NaCl (right). (Photo: Sohail Ehsanzamir, SLU)

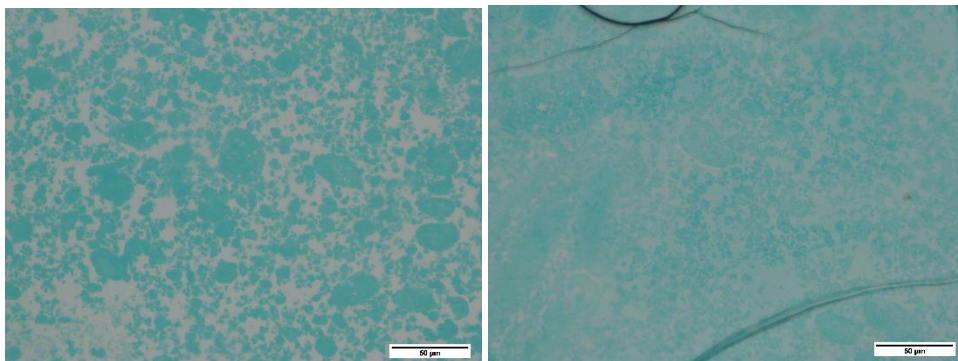


Figure 6. APH5 (left) and APH5 + NaCl (right). (Photo: Sohail Ehsanzamir, SLU)

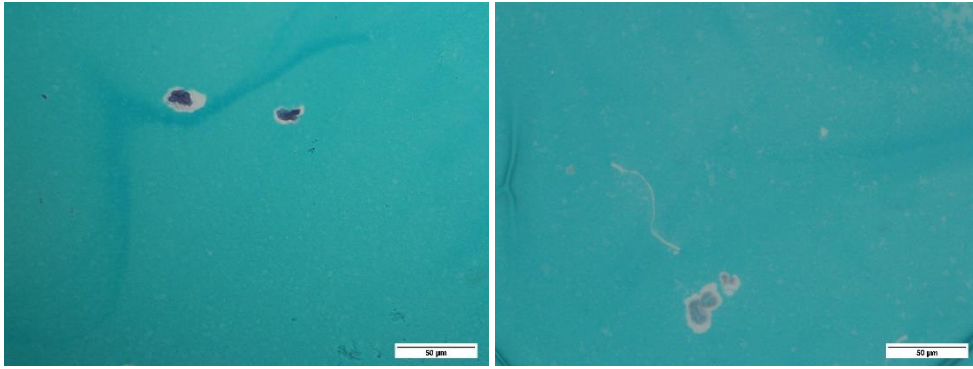


Figure 7. BMPH7 (left) and BMPH7 + NaCl (right). (Photo: Sohail Ehsanzamir, SLU)

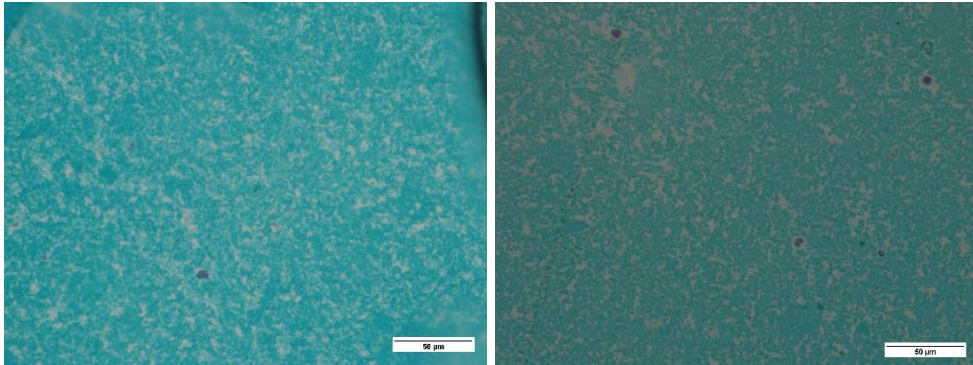


Figure 8. BMPH5 (left) and BMPH5 (right). (Photo: Sohail Ehsanzamir, SLU)

3.4 Compression test

The depicted stress- strain curves obtained from compression tests does not show the average of curves for all samples. Instead a curve which was representable for respective sample was chosen. The average values for the samples are presented in table 7. Results from compression tests on gels made from bean milk extracts shows that BMPH5 and BMPH7 made the hardest gels and the addition of NaCl to these weakened them (figure 9).

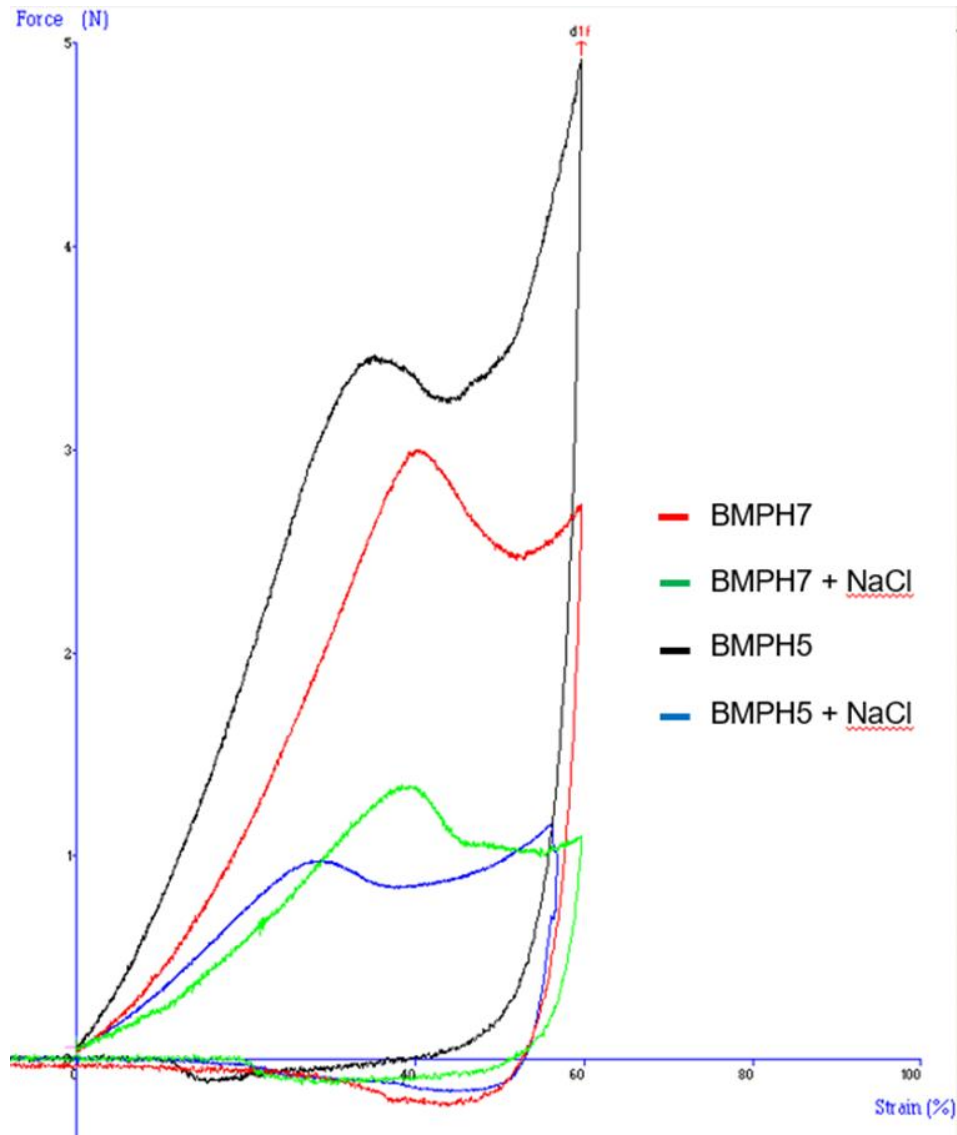


Figure 9. Stress- strain curves for gels from bean milk extracts.

Figure 10 shows representative curves of gels made from alkali extracts. APH7 made the hardest gels followed by APH7 + NaCl, which indicates that NaCl does weaken the gel at pH 7. At pH 5 however, NaCl had no significant effect and both APH5 and APH5 + NaCl lacked fracture points. pH 5 gels also reached yield point at a lower strain.

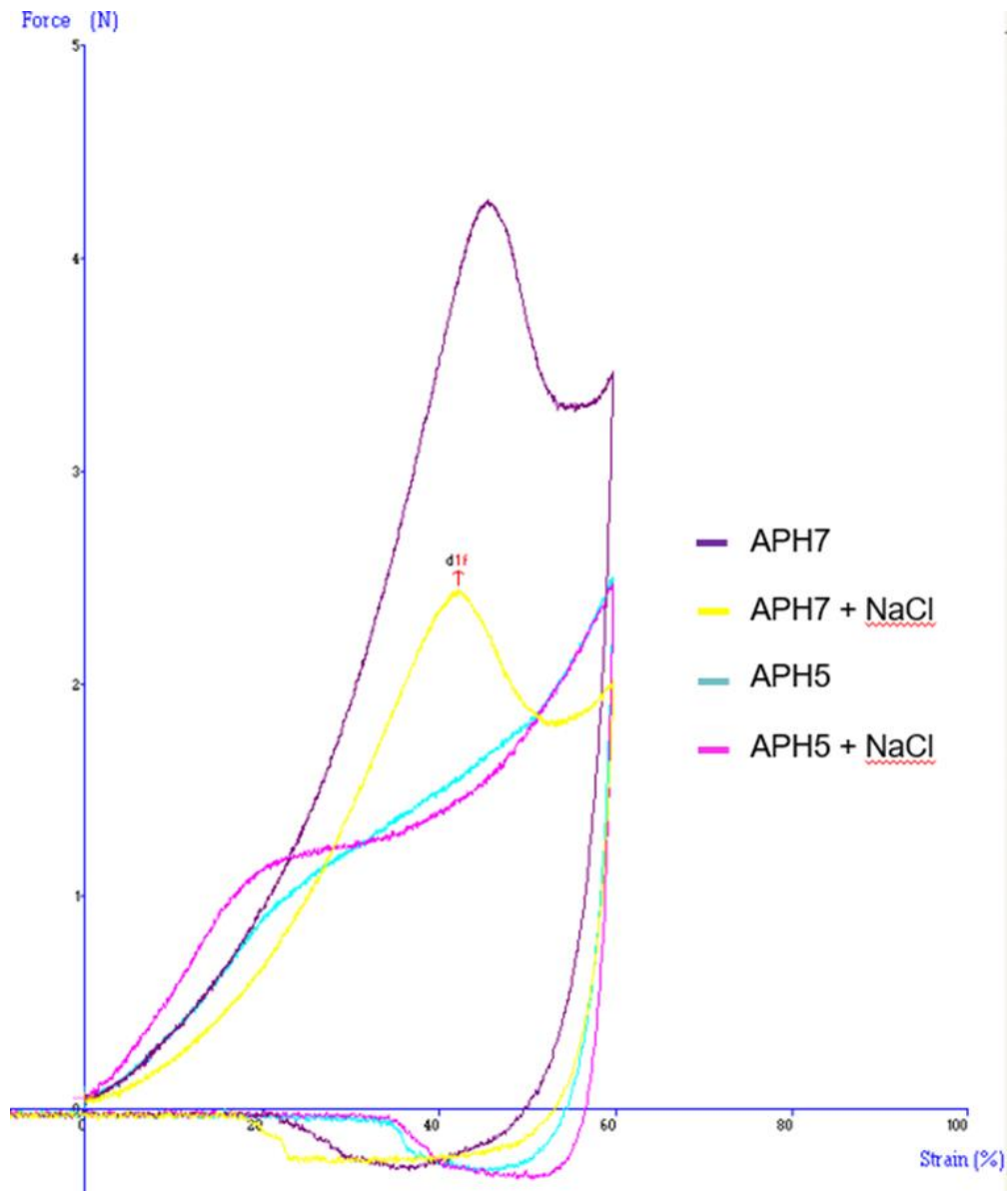


Figure 10. Stress- strain curves for gels from alkali extracts.

3.5 Statistical analysis

Results of compression tests are summarized in table 7 with means and standard deviations. The sample variances are plotted in figure 11- 14.

Sample hardness is read from the column “Fracture strength” and elasticity in “Yield Point”, which in this case are correlated to each other. Stiffness in “Young’s modulus” and cohesiveness in “Yield Point Strain”. The one- way ANOVA test gave a *p*- value of <0.001 among the means at the 0.05 level of significance, for every parameter. Hence it can be concluded that there is a significant difference among the means. However, all samples were not significantly different each other and is also shown in table 7.

pH 7 gels formed the overall hardest ones and those from alkali isolates were slightly harder and showed better cohesiveness than those from bean milk extracts. However, the addition of NaCl weakened both APH7 and BMPH7 significantly. NaCl also changed the cohesive properties of APH7 but did not have any effect on APH5 and BMPH7. At pH 5, BMPH5 made the hardest gels and were also more cohesive than APH5.

Table 7. Means and standard deviations of gels for different parameters. Variables that share any letter in the same column do not significantly differ from each other

Name	Fracture strength (KPa)	Young’s modulus (KPa)	Yield point (KPa)	Yield point strain (%)
APH7	49 ± 9.2 ^e	93.6 ± 18.7 ^{fg}	40.4 ± 10.2 ^{gh}	42.8 ± 2.3 ^f
APH7 + NaCl	23.8 ± 1.7 ^c	56.4 ± 5.6 ^{bcd}	19.2 ± 2.9 ^{de}	33.8 ± 2.7 ^{de}
APH5	-	48.4 ± 8.1 ^{ac}	9.9 ± 1.9 ^a	20.5 ± 2.8 ^{ab}
APH5 + NaCl	-	60.2 ± 9.4 ^{bce}	11.0 ± 2.4 ^a	18.2 ± 2.3 ^a
BMPH7	30.5 ± 2.9 ^d	76.6 ± 7.7 ^{ef}	27.7 ± 3.2 ^{fh}	36.0 ± 2.2 ^e
BMPH7 + NaCl	15.4 ± 2.1 ^a	39.2 ± 4.3 ^a	14.2 ± 1.9 ^{ad}	36.4 ± 2.5 ^e
BMPH5	34.1 ± 10.3 ^{cde}	105.7 ± 28.2 ^{deg}	31.6 ± 10.6 ^{efg}	30.5 ± 2.2 ^d
BMPH5 + NaCl	10.4 ± 3.3 ^a	38.7 ± 10.9 ^{ab}	9.6 ± 3.2 ^a	24.4 ± 1.8 ^{bc}

APH7, BMPH7 and BMPH5 gave the overall hardest and stiffest gels but with a large variance between samples, especially in APH7 and BMPH5. BMPH5 + NaCl, APH5 and APH5 + NaCl resulted in the weakest gels followed by APH7 + NaCl. The addition of 2% NaCl weakened all gels except for in APH5.

APH5, APH5 + NaCl and BMPH5 + NaCl also reached yield point at lowest strain.

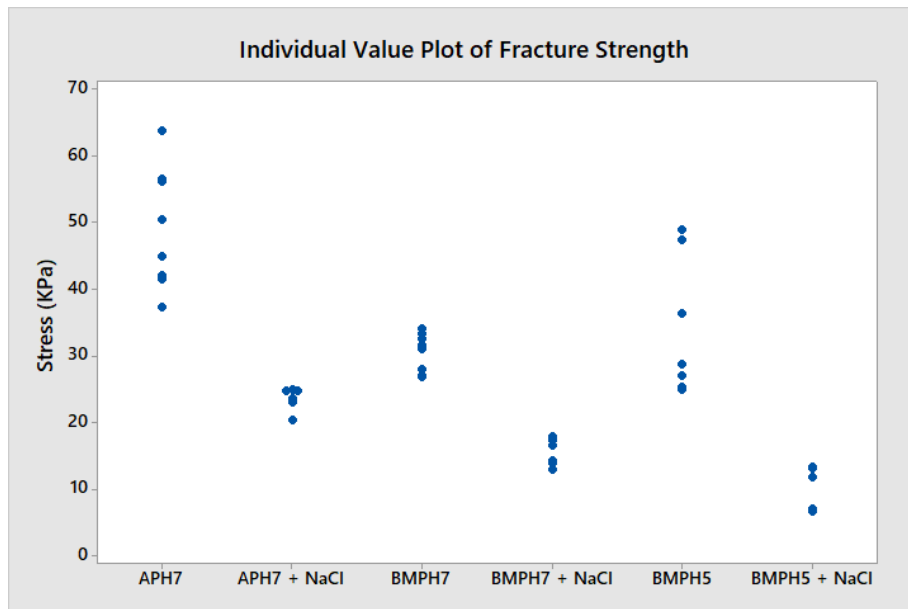


Figure 11. Sample variance are plotted for those gels that showed a fracture point.

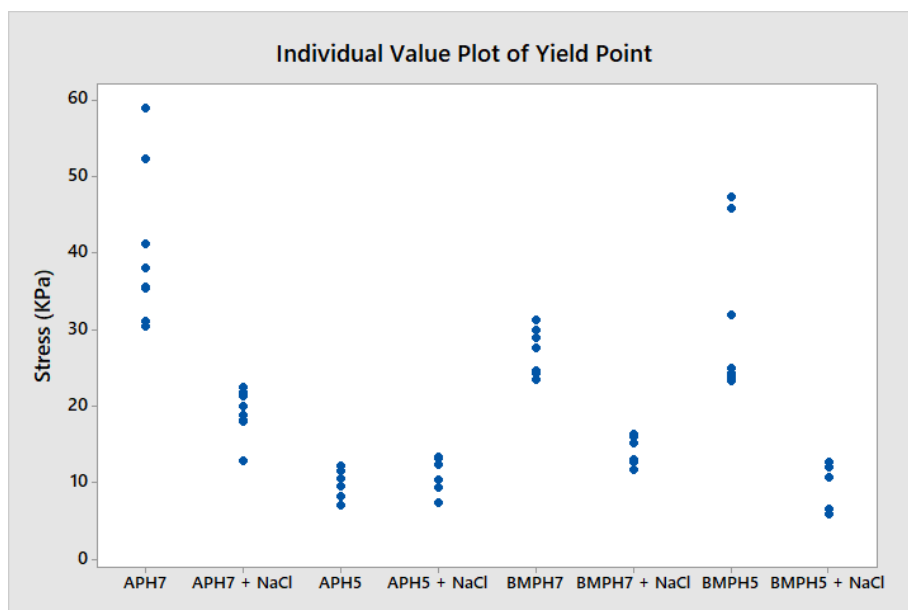


Figure 12. Sample variance is plotted for the point at which plastic deformations starts.

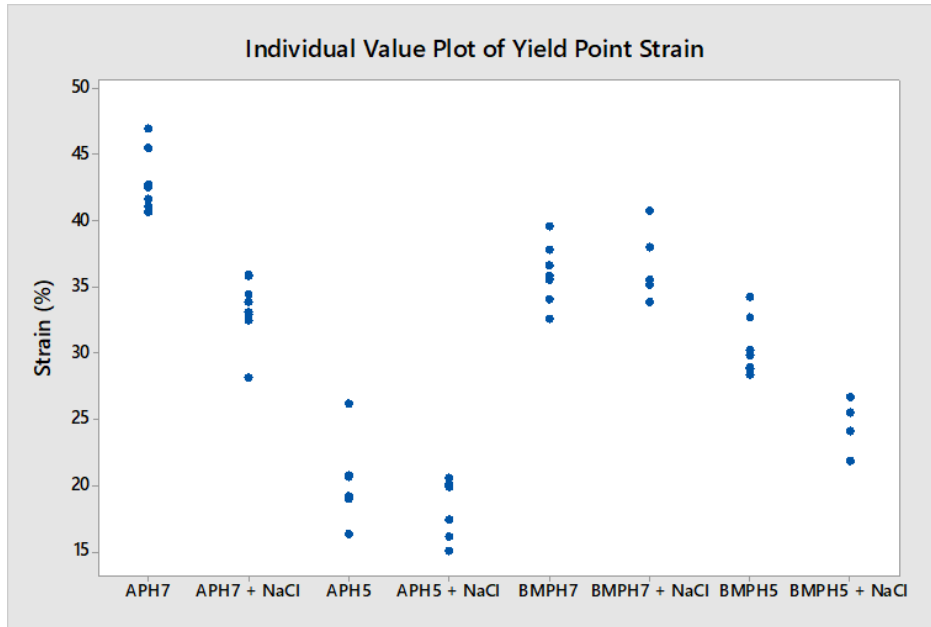


Figure 13. Sample variance are plotted for the strain at which gels reach yield point.

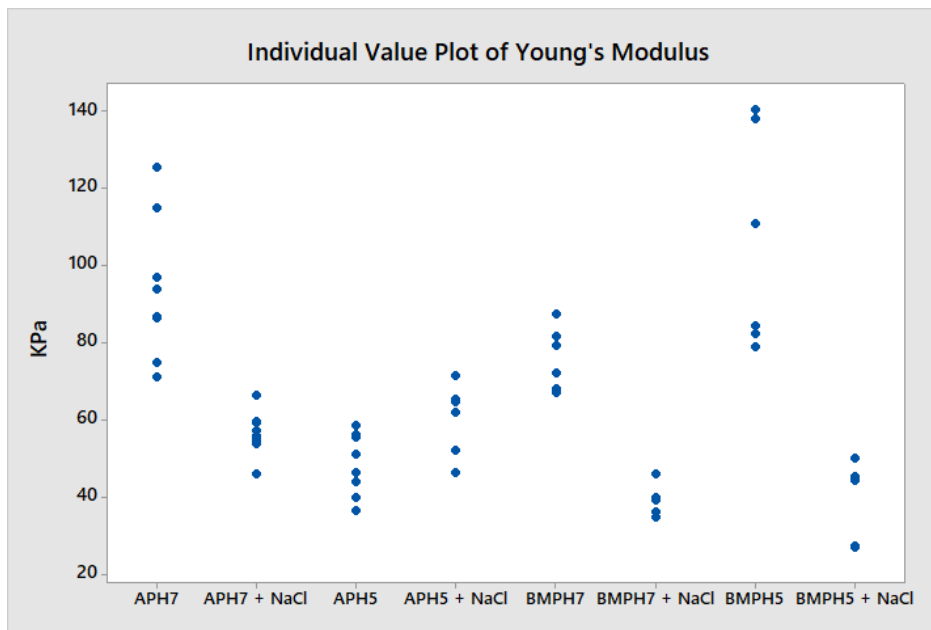


Figure 14. Sample variance plotted for Young's modulus.

4 Discussion

Methods and materials were designed in such way to be applicable in food industries. The protein isolates, after extraction and in water solution are susceptible to pathogens and oxidation and isolates were therefore freeze-dried to increase the shelf-life. Also, freeze-drying the isolates provides the possibility to add proteins in desired concentration. Sodium chloride was selected because it is the most common salt additive in households and in food production.

Protein concentration in freeze-dried isolates was determined according to Kjeldahl method by using 5.4 as the conversion factor. Earlier studies have reported that 5.4 is more representable than 6.25 for calculating protein content in legumes (Mariotti *et al.*, 2008). Calculating the protein content of dehulled Gloria flour and comparing results to the concentration reported earlier, indicates the reliability of the method. The estimated protein content from the Kjeldahl procedure was 29.4% (table 4), which is close to reported earlier; 30%. Using 6.25 as the conversion factor for determining protein content in legumes or at least fava beans, provides an over-estimation. On the assumption that the general conversion factor 6.25 were used instead, the protein content of Gloria flour would be closer to 37% when corrected for dry content.

As earlier mentioned, the least gelling concentration varies for different gelling agents and is highly influenced by pH, concentration and ionic strength, which is why the estimated LGC for all samples varied significantly. The general observation was that proteins adjusted to pH 5 had lower LGC and the addition of 2% sodium chloride lowered it further. Chen and others also reported a decreased LGC for a given heat treatment when pH was lowered in soybean protein (Chen *et al.*, 2017a).

Protein isolates from bean milk had an overall lower LGC than those from alkali extract, suggesting that other compounds affect the gelation of bean milk proteins. Another possible explanation for this difference is that the different extraction methods extracts different fractions of proteins. However, both alkali and bean milk isolates, independent on pH or sodium chloride content, did not form a firm gel until

12- 14% proteins (w/w). Fernández- Quintela and others (1997) obtained a LGC of 14% for protein isolates from fava beans, but used the conversion factor 6.25.

Based on these results, a protein concentration of 15% were chosen for the texture- and microscopy analysis.

As already described; proteins with added sodium chloride had lower LGC than those without, however the stress- strain curves from compression tests showed that at a concentration of 15% proteins, 2% NaCl weakened all gels except in APH5 where it had no influence in particular (table 7). The results are consistent with the study of Makri and others (2006), where the addition of 0.25 mol L⁻¹ NaCl weakened gels made from fava bean proteins. Adding NaCl to the protein- gels has the same effect as adjusting the pH closer the isoelectric point. The increased ionic strength lowers the solubility of proteins and affects the aggregation of vicilin, legumin and others. This property explains why gels were generally weakened by 2% NaCl and lower pH (Chen *et al.*, 2017a).

The reason that the obtained stress- strain curves from APH5 and APH5 + NaCl did not show any fracture points is due to lack of cohesiveness and rather than fracturing it is mashed. The bending of the curve gives the false pretences that it is a ductile material when in reality it is the result of its low cohesiveness. Microscopy analysis of APH5 shows that the distance between aggregates are so extensive that the addition of NaCl may not exhibit any significant effect on these (figure 6). Even though the curves could not directly give a fracture strength for APH5 and APH5 + NaCl, we can compare fracture strength to yield point and notice a positive correlation between them.

Micrographs shows that bean milk isolates contain more impurities such as starch and cell walls than alkali extracts. These results were expected due to the higher protein content in alkali extracts. Otegui and others (1997) made similar alkali extracts and obtained residuals such as fat (3.9%) and carbohydrates (8.4%). These have the ability to interfere with the interactions between proteins and water but also between proteins. They mainly alter the water binding properties by changing the accessibility of the polar amino groups of proteins that are the primary sites for protein- water interactions. As mentioned earlier, present starch granules may also take up water and swell. Studies investigating the relationship between starch granules and protein gels are warranted because it may be the cause to why BMPH7 is weaker compared to APH7. Both BMPH7 and APH7 gels form a continuous protein phase but the former's phase is interrupted by starch granules and cell walls more often. There is no visible effect of NaCl addition on APH5- gels, neither did compression tests show any difference. But the addition of NaCl to BMPH5 increases the distance between proteins, which results in lower cohesiveness. This is further supported by results from compression tests, where BMPH5 + NaCl proved to deform plastically (yield point) at an earlier strain.

The difference between BMPH5 and APH5 is surprising. Stress- strain curves (figure 9 & 10) shows that BMPH5 is harder and have better cohesive properties. The difference is also visible in a light microscopy where aggregates of BMPH5 is more densely packed compared to APH5 (figure 8 & 6). A larger part of the isolates from the bean milk are unknown and these might have an effect in gel hardness, but micrographs suggests that it is the protein fraction, which may exhibit the most significant difference. Cai and others (2002) studied the relationship between textural properties of legume curds and protein fractions. They reported that higher 11S fraction in globulins increased hardness, springiness and cohesiveness of curds. The ratio of 11S to 7S may be higher in the bean milk isolate than in the alkali isolate and provide this stability at pH 5. Isolates from bean milk would therefore be preferred in e.g. the production of harder cheeses. According to the strain at which gels reaches its yield point (figure 13), NaCl seems to only lower cohesive properties in APH7 and BMPH5 but not BMPH7. Apparently, it somehow weakens the gel, makes it more flexible but does not affect its cohesive properties.

Results from this study displays that fava beans can be used as a whole or as an additive to increase protein content while maintaining the same texture due to its good gelation properties. Also, gels can be altered to gain the wanted texture by using isolates from different extraction methods or changing pH and sodium chloride content. However, it has a distinct smell and taste, which needs to be altered with to help in consumer's acceptance.

4.1 Limitations

The results are indicative but limited to a certain point. The addition of sodium chloride did in this study show to weaken almost all gels, but it would be a mistake to conclude that any addition of sodium chloride generates equivalent results. To draw such conclusion, one must perform tests with NaCl gradients. Also, due to pH adjusting with both NaOH and HCl, both Na⁺ and Cl⁻ ions are already present before any addition of NaCl. The increase in ionic strength which NaCl provides may alter the interactions of proteins and affect results, especially in pH 5 gels where more HCl is required. Ionic strength has earlier been reported to affect the solubility of fava bean proteins. Arogrundade and others observed that increasing ionic strength with NaCl to 0.05- 0.4 increased protein solubility but decreased again thereafter.

Furthermore, there was a large sample variance. Many parameters could alter the results. One of those is the speed of the magnetic stirrer during pH adjustment. It was noticed that higher rpm during the initial stages of gel production lead to more incorporated air in the dispersion. When further studies on the gelation properties of these isolates are performed, the speed of the magnetic stirrer should be

standardized. Another option is to degas dispersions in a vacuum desiccator before heat treatment.

4.2 Conclusion

Gelation is a complicated phenomenon and a gelling agent's gelation properties is highly influenced by the presence of other compounds, which may alter the gel strength and cohesiveness. The protein isolates extracted from fava beans in this study performed differently when altering parameters such as pH, protein concentration and NaCl content. The isolates from the alkali extraction made the hardest and stiffest gels at pH 7. They were also more cohesive. The opposite was observed at pH 5, where isolates from the bean milk extraction had better gelation properties. Residuals in respective protein isolate may be involved in the difference. Another difference may lie in the protein fractions that are extracted from each method. However, both isolates made weaker gels as NaCl were added.

Fava beans and isolated proteins from them have the potential to replace imported soybean, both as feed and food for human consumption and the advantages are both economically and environmental. Soybeans grown in e.g. Brazil are treated with pesticides, which have been prohibited in Sweden for a long time due to their negative effect on human health. Also, domestic production of fava beans would decrease greenhouse gas emissions from transports. Fortunately, Sweden has excellent soil and climate for growing of fava beans. There has been an increased production the recent years but mainly for usage as animal feed. It has yet to be introduced in the food industry.

Findings from this study can be applied in food production when trying to enhance nutritional quality but maintaining the same texture or when making e.g. cheese from fava bean proteins. Yet, further research on the protein fractions and residuals in the protein isolates are warranted to fully understand the effect of involved components.

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Appendix 1: Popular scientific summary

Gelation is a vital phenomenon in foods. Gelation of proteins from fava beans has been studied by changing pH and sodium chloride content to evaluate its potential as food.

Fava beans (*Vicia Faba*) is a legume that is mainly used as feed for cattle. It's high protein content makes it also interesting to use as food for humans as we are searching for a sustainable protein source. In Sweden, fava beans have the potential to replace soybeans due to its ability to grow well in the Swedish soil and climate. Although, studies that investigates its functionalities are warranted and already several companies are on their way.

Gelation is a phenomenon, which can be altered with by changing various parameters and the resulting gel is of great importance for the texture of foods. Proteins from fava beans form gels that are firm at a neutral pH and also at lower, but it depends on which method that was used for the extraction of proteins. However, one of the most common additives used both in the kitchen and the industry; sodium chloride, have the ability to weaken those gels significantly. Practically it means that fava beans can be used in foodstuff to obtain different textures by changing sodium chloride content. If sodium chloride is added, one may expect that the texture will be softer than in the same foodstuff but without sodium chloride.

Currently, large studies are being conducted on the potential of fava beans as food but there is one major concern; the aroma. It has a distinctive smell and taste which needs to be removed. Fortunately, there are methods that are available, such as deodorization. This removes compounds responsible for the aroma, which will hopefully aid in the consumer's acceptance.