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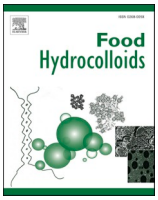

Effect of starch and fibre on faba bean protein gel characteristics

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ABSTRACT

Faba bean is a promising alternative to soybean for production of protein-rich plant-based foods. Increased understanding of the gelling behaviour of non-soy legumes can facilitate development of novel plant-based foods based on other legumes, such as faba bean. A mixture design was used in this study to evaluate the effect of different proportions of protein, starch and fibre fractions extracted from faba beans on gelation properties, texture and microstructure of the resulting gels. Large deformation properties, in terms of fracture stress and fracture strain, decreased as fibre and/or starch replaced protein. In contrast, Young's modulus and storage modulus increased with substitution of the protein. Light microscopy revealed that for all gels, protein remained the continuous phase within the region studied (65–100% protein fraction, 0–35% starch fraction, 0–10% fibre fraction in total flour added). Swollen and deformed starch granules were distributed throughout the mixed gels with added starch. Leaked amylose aggregated on starch and fibre surfaces and in small cavities (<1 μm) throughout the protein network. No clear difference between samples in protein network structure was observed by scanning electron microscopy. The reduction in large deformation properties was tentatively attributed to inhomogeneities created by the added starch and fibre. The increase in small deformation properties was hypothesised to be affected by water adsorption and moisture stability through the starch and fibre, increasing the effective protein concentration in the surrounding matrix and enhancing the protein network, or potentially by starch granules and fibre particles acting as active fillers reinforcing the gel structure.

1. Introduction

The food sector contributes significantly to the global environmental impact, accounting for e.g. 34% of total greenhouse gas emissions in 2015 (Crippa et al., 2021). Increasing consumption of locally produced plant-based foods have the potential to reduce the environmental impact of food consumption (Willett et al., 2019). Legumes, such as faba beans, can serve as good plant-based protein sources. Faba beans can be grown in most climate areas of Europe, including Sweden, where they are extensively produced but mainly used for animal feed (Crépon et al., 2010; Jansson, 2019). Faba beans are rich in protein, starch, fibre and micronutrients (Crépon et al., 2010). The major proteins in faba beans are the globular storage proteins legumin (11S) and vicilin (7S) (Warsame, O'Sullivan, & Tosi, 2018).

Protein gelation is an essential step in production of a range of foods. Today, many protein-rich plant-based foods are based on soy, but faba beans can serve as an alternative protein source, with promising properties in e.g. production of tofu (Jiang, Wang, Stoddard, Salovaara, & Sontag-Strohm, 2020). Protein gelation can be affected by multiple factors, such as protein source, pH, salt and extraction method (Nicolai & Chassenieux, 2019). The effect of extraction method has been investigated previously in terms of dry fractionation and dehulling on the functionality of faba bean protein fractions (Saldanha do Carmo et al., 2020; Vogelsang-O'Dwyer et al., 2020). It has been shown that dry fractionation has a lower environmental impact than wet fractionation, but yields less pure fractions (Vogelsang-O'Dwyer et al., 2020). Wet extraction and isoelectric precipitation have the benefit of almost completely removing the favism-causing glucosides vicine and convicine (Vioque, Alaiz, & Girón-Calle, 2012). Investigations of the effects of different wet protein extraction methods (alkali extraction and soaked extraction) on the texture and microstructure of faba bean protein gels have shown that gelation at neutral pH, compared with slightly acidic

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pH 5, results in less coarse gels (Langton et al., 2020).

Most foods have complex matrices containing more than one component. In addition to protein, faba beans also contain significant amounts of starch (40–44%) (Crépon et al., 2010). Protein and starch can be combined to create mixed gels, which have been studied previously for other protein and starch sources (Aguilera & Baffico, 1997; Joshi, Aldred, Panozzo, Kasapis, & Adhikari, 2014). The contribution from starch has been found to differ depending on whether the starch gels before or after the protein (Muhrbeck & Eliasson, 1991). If the starch in a mixed protein/starch gel is allowed to gel before the protein, the starch and protein will form two separate networks, supplementing each other without any specific interactions (Muhrbeck & Eliasson, 1991). If the starch has a higher gelatinisation temperature and the protein gels first, diffusion and network formation by the starch amylose is hindered and the starch instead acts mainly as a filler material. Studies on β-lactoglobulin/amylopectin gels have shown that amylopectin can influence protein aggregate density and connectivity at different length scales as observed by light microscopy (LM), confocal laser scanning microscopy (CLSM) and transmission electron microscopy (TEM) (Olsson, Langton, & Hermansson, 2002). These microstructural changes are correlated to the rheological properties of the gels (Olsson et al., 2002).

Another component present in significant amounts in faba beans is fibre (9–10% crude fibre) (Crépon et al., 2010). Dietary fibre is important for human health and can have multiple health benefits (Stephen et al., 2017). Nonetheless, average dietary fibre intake in most European countries is estimated to be below the recommended level (Stephen et al., 2017). Many side-streams from the food industry are rich in fibre and can be added to different food systems to modify their texture and functional properties (Lan et al., 2020; Niu et al., 2021). Faba beans contain mainly insoluble fibre (10.7–16.0%) and minor amounts of soluble fibre (0.6–1.1%) (Mayer Labba, Frøkiær, & Sandberg, 2021). Insoluble dietary fibre (IDF) from sugarcane has been shown to improve myofibrillar protein gelation properties through moisture stabilisation, due to its high water-holding capacity (Zhuang, Jiang, et al., 2020).

As food matrices are often complex, it is important to understand the effect of different macromolecules on the gelation properties of protein, particularly as dry fractionation, where the resulting fraction is less pure than in wet extraction, is increasing in popularity. In this study, the effect of starch- and fibre-rich fractions on faba bean protein gelation was investigated using a mixture design approach. This approach is widely applied in product formulation and can be used to study two or more components mixed in different ratios (Buruk Sahin, Aktar Demirtaş, & Burnak, 2016; Lawson, 2014). The effect of changes in the ingredient ratios can be studied and interaction effects can be identified. Surface or contour plots can be used to visualise how different product properties are influenced by the ratios of the raw materials.

The aim of this study was to determine the effect of substituting part of the protein for starch- and/or fibre-rich fractions on gel texture and microstructure in faba bean-based protein gels. Gel texture was analysed in terms of small and large deformations and microstructure, using light and scanning electron microscopy (SEM).

2. Materials and methods

2.1. Materials

The faba beans (*Vicia faba* L. var. *Gloria*) used for extraction were kindly provided by RISE (Research Institutes of Sweden). The beans were grown in central Sweden and harvested and dried in 2016. Before use in this study, the faba beans were dehulled (Hi-Tech Machinery Manufacturing Co. Ltd., China), separating the cotyledon and hull, and milled (Ultra-Centrifugal Mill ZM-1, Retsch, Germany) into flours using a mesh size of 0.5 mm. Only the cotyledon flour was used for further extraction.

NaOH and HCl were purchased from Merck KGaA (Darmstadt, Germany), iodine, glutaraldehyde, ruthenium red and light green from Sigma-Aldrich (St. Louis, MO, USA), Technovit 7100 from KULZER (Hanau, Germany) and osmium tetrahydroxide from Ted Pella (Redding, CA, USA). Chemicals and sugar standards used for analysis of monosaccharides and uronic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Extraction of protein, starch and fibre fractions

The extraction process used to obtain the different faba bean fractions was based on isoelectric precipitation of protein and a starch extraction procedure described previously, with some modifications (Lee, 2007).

To extract the protein, the faba bean cotyledon flour (200 g) was dispersed in distilled water at a ratio of 1:10 (w/v). The pH was adjusted to 9.0 using 2 M NaOH and the dispersion was stirred for 1 h at room temperature before centrifugation (Thermo Scientific, Sorvall Lynx 4000, Waltham, MA, USA) at 3700×*g* (20 °C, 30 min). The supernatant was collected, pH-adjusted to 4.0 (a value chosen based on protein yield; (Herneke et al., 2021)) using 1 M HCl, stirred at room temperature for 1 h and then centrifuged at 3700×*g* (20 °C, 15 min). The pellet was collected and washed once by dispersion in distilled water at a ratio of 1:10 (w/v) with pH adjustment to pH 4.0, followed by centrifugation at 3700×*g* (20 °C, 15 min). The pellet was collected and freeze-dried (Martin Christ, Epsilon 2-6D LSC Plus, Osterode am Harz, Germany).

The starch and fibre fractions were extracted from the pellet obtained from the first centrifugation step during protein extraction. The pellet was dispersed in 750 mL 33.3 mM NaOH and stirred at room temperature for 24 h. The mixture was then left to stand, without agitation, at 4 °C for an additional 24 h before centrifuging at 3700×*g* (20 °C, 5 min). The resulting supernatant was discarded and replaced with distilled water. The washing process was repeated approximately five times until pH 7 was reached. After the final wash, the pellet was collected and mixed in a kitchen blender (Wilfa, XPLODE Vital, Hagan, Norway) with 1 L distilled water for 2 × 20 s on the 'smoothie' setting. Thereafter, the mixture was filtered through a 70 μm nylon filter. The filter cake was dispersed in 600 mL distilled water, mixed in the kitchen blender as previously described and filtered through the nylon filter. The mixing and filtering process was repeated 12 times, until further washing only recovered minor amounts of starch per additional washing step. After the last filtering step, the filter cake was collected and freeze-dried to obtain the fibre fraction. The filtrate was stored overnight without agitation at 4 °C to allow the starch to sediment. The supernatant was decanted and the sedimented starch dried in an oven at 40 °C for 48 h, or until fully dry. All three fractions were ground into flours using a pestle and mortar before use. The starch and fibre fractions were sieved (Retsch, AS200 basic, Haan, Germany) using a 250 μm mesh to remove any larger particles.

2.3. Characterisation of raw materials and extracted fractions

The analysis of protein, fat, neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin content was performed by the Analysis Laboratory at the Department of Animal Nutrition and Management, Swedish University of Agricultural Sciences, Ultuna. The crude protein content was analysed using the Kjeldahl method and a conversion factor of 5.4 (Mosse, 1990; Nordic Committee on Food Analysis, 1976). The measurements were performed using the digestion system Digestor 2520 combined with a Kjeltec 8400 analyser unit and an 8460 sampler unit (Foss Analytical A/S, Hillerød, Denmark). NDF and ADF were measured as described previously (Chai & Udén, 1998; Van Soest, Robertson, & Lewis, 1991). The fat content was determined according to the EU guideline method (EC) 152/2009 (Commission Regulation (EC) No 152/2009. H. Determination of Crude Oils and Fats, 2009), using a Hydrotec 8000 in combination with a Soxtec Extraction unit (Foss Analytical A/S, Hillerød, Denmark). For the fibre fraction, NDF, ADF, lignin, cellulose and hemicellulose were analysed using sequential

analysis (Robertson, Van Soest, James, & Theander, 1981), whereas the ADF and NDF in the protein and starch fractions were analysed separately (Chai & Udén, 1998; Van Soest et al., 1991). The cellulose content in the cotyledon and hull flours was calculated as the fraction remaining after subtracting the starch, protein, fat, hemicellulose, lignin and ash. All measurements were performed in at least duplicates, except for the protein and fat content in the cotyledon and hull flours, where only one sample was analysed.

Analysis of starch, resistant starch, moisture and ash content was performed in duplicate. Total starch content was determined using a Total Starch Assay Kit (Total starch HK assay kit, Megazyme Ltd, Wicklow, Ireland). Resistant starch was determined using a Resistant Starch Assay Kit (K-RSTAR, Megazyme Ltd, Wicklow, Ireland). Moisture content was determined by oven drying at 105 °C overnight and ash content according to the AOAC official method 942.05.

The monosaccharide composition of the faba bean cotyledon, hull and extracted fibre fraction was determined using a modified version of an existing method (Sluiter et al., 2008). First, 3 mL 72% H_2SO_4 was added to 200 ± 0.5 mg of sample in a 150 mL beaker and kept under vacuum for 15 min, before being placed in a water bath for 1 h at 30 °C with stirring every 20 min. Thereafter, 84 g of deionised water were added, followed by autoclaving at 125 °C for 1 h. The samples were filtered through a 1.6 μm filter (Whatman Grade GF/A Fine Retention Filter) with vacuum and diluted to a total volume of 100 mL. The filtrate was further diluted (1:10) with deionised water to remain within the calibration range, and fucose was added as an internal standard to obtain a total concentration of 400 mg fucose/L. Samples were then filtered through 0.2 μm filters (Acrodisc Syringe Filters with PVDF Membrane) into HPLC vials. The monosaccharide composition was analysed using an ion chromatograph (ICS 3000 Dionex) equipped with an AEC column (CarboPac PA 1 analytical 4 × 250 nm). Standards used were D (+) glucose, D (+) xylose, D (+) galactose, L (+) arabinose, L (+) rhamnose and D (+) mannose. All samples were measured in duplicate.

Uronic acid analysis was performed according to the Blumenkrantz-Asboe-Hansen method (Blumenkrantz & Asboe-Hansen, 1973) with modifications. In short, 10.6 ± 0.4 mg sample was weighed into a 10 mL glass tube. The samples were kept on ice and 0.5 mL 96% H_2SO_4 was added twice with 5 min of intermittent vortexing, after which 0.25 mL of deionised water was added twice with 5 min of intermittent vortexing. Deionised water was then added to a total volume of 10 mL. Next, 20 μL of 4 M sulfamic acid-potassium sulfamate (pH 1.6) were added to 1.5 mL plastic Eppendorf tubes, followed by 160 μL of the hydrolysed sample and 800 μL 12.5 mM Borax in 96% H_2SO_4. The tubes were oven-incubated at 95 °C for 20 min and the samples were cooled and pre-read on a UV–Vis spectrophotometer (Cary 60 UV–Vis Spectrophotometer, Agilent Technologies) at 525 nm, followed by addition of 40 μL of 0.15% (w/v) 3-phenylphenol in 0.5% (w/v) NaOH and reading again at 525 nm. A standard curve was created using D (+) galacturonic acid. The pre-read value was subtracted from the absorbance read after the colorimetric reaction to obtain the uronic acid concentration. All samples were analysed in duplicate.

2.4. Experimental design

An extreme vertices mixture design including axial blends and the overall centre point was used for analysis of small and large deformations (Fig. 1) (Lawson, 2014). Two additional mixtures, not included in the original design, were included along the protein-starch and protein-fibre axis for further investigation of the two-component systems. A total solids content of 20% was used unless otherwise stated. Of these 20% solids, the proportion of protein fraction (X_1) was kept in the range 65–100%, the starch fraction (X_2) was limited to 0–35% due to sedimentation and the fibre fraction (X_3) was limited to 0–10% due to layering during mixing at higher fibre content. The exact percentages of protein, starch and fibre fraction at each design point are listed in Table S1. Rheology experiments were run with two replicates at each design point. One replicate (mean value of 5–8 gels produced simultaneously from the same batch) was run at each point for the compression test, except for the centre point, at which six replicates were run to assess the variance.

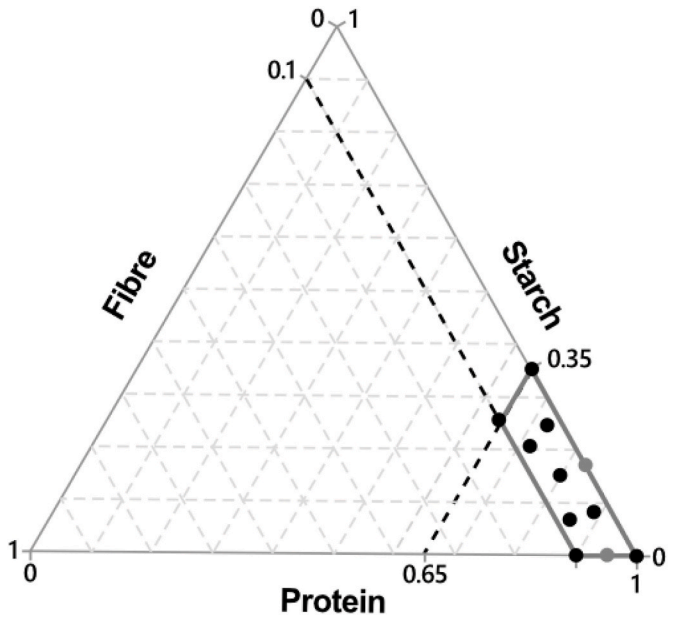

Fig. 1. The extreme vertices mixture design, including axial and centre points, used for the experiments with two additional experimental points (grey) added along the two-component axes.

2.5. Gel formation

Ingredients were dry mixed before being dispersed in distilled water and stirred for 15 min, followed by adjustment of the pH to 7.0 using 2 M NaOH. After pH adjustment, the mixture was stirred for 30 min with additional pH adjustments when needed and volume adjustment to give a final concentration of 20% (g flour/g sample). To limit sedimentation of the starch, the mixture was stirred for an additional 20 min in a water bath at 58 °C. Thereafter, 2.8 mL were loaded into hollow glass tubes with inner diameter 12 mm. The bottom of the glass tubes was closed with a rubber lid and the top with thread tape punctured with a small hole to prevent pressure build-up. The samples were heated in a water bath at 95 °C for 30 min, then cooled in tap water and stored in a fridge overnight for compression test and preparation for microscopy.

2.6. Rheology

A Discovery HR-3 rheometer (TA Instruments, New Castle, DE, USA) equipped with a 40 mm aluminium plate was used to study the gelation process of mixtures prepared as described in section 2.5, excluding the final heating step at 95 °C (Sun & Arntfield, 2011). Paraffin oil, coating the exposed parts of the sample, combined with a custom made solvent trap was used to limit sample evaporation (Saldanha do Carmo et al., 2020). Storage modulus (G') and loss modulus (G'') were recorded at a frequency of 1 Hz and a strain of 0.5% during a temperature ramp. A strain sweep was performed on each sample after gelation to confirm that the strain was within the linear viscoelastic region. The temperature ramp comprised heating from 60 to 95 °C at a rate of 1.5 °C/min followed by a holding time at 95 °C for 30 min and cooling to 25 °C at 1.5 °C/min followed by an additional holding time of 30 min at 25 °C.

2.7. Compression tests

Samples prepared as described in section 2.5 were allowed to

equilibrate to room temperature for 1 h before being cut into cylindrical pieces with height 14 mm and diameter 12 mm. Compression tests were performed using a texture analyser (Stable Micro Systems, TA-HDi, Surrey, UK) equipped with a 500 N load cell and a 36 mm cylindrical aluminium probe. The samples were compressed to 60% at a rate of 1 mm/s. True stress and true strain were determined as described previously (Munialo, van der Linden, & de Jongh, 2014). True fracture stress and true fracture strain (hereafter referred to simply as fracture stress and fracture strain) were defined as the maximum true stress and corresponding true strain at the first clear peak before fracture. Young's modulus was calculated as the slope of the true stress-true strain curve during the initial 1–5% of deformation.

2.8. Regression analysis of compression tests and rheology

Regression analysis using the standard least squares method in JMP Pro version 16.0.0 (SAS Institute, Cary, NC, USA) was performed, fitting the data to a reduced version of the special cubic Scheffé model:

$$Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{123} X_1 X_2 X_3 \quad (1)$$

where Y is the predicted response, X_1, X_2 and X_3 are the protein, starch and fibre content given as proportions of the total amount of each fraction added, β_1, β_2 and β_3 are the regression coefficients for each linear effect term, and β_{12}, β_{13}, β_{23} and β_{123} are the regression coefficients for the binary and ternary interaction effects terms. The L-pseudo component scaling in JMP was used for rescaling the components and to reduce multicollinearity. A dummy variable technique (Miller, 1984; Redgate, Piepel, & Hrma, 1992), combined with forward selection to minimise the Akaike information criteria (AIC), was used to decide what terms to include in the final model. In short, random and uniformly distributed dummy variables were generated and added to the list of potential candidate variables for the model. The selection process was stopped when either the first dummy variable entered the model or the AIC was minimised. The main effect terms (β_1, β_2 and β_3) were forced into the model. After model fitting, residuals were checked for homoscedasticity and normal distribution. Adjusted coefficient of determination ($R^2(adj)$) was used to determine how well the model fitted the sample data and the lack-of-fit was tested to evaluate whether the model adequately described the functional relationship between the experimental factors and the response variable. Contour plots of raw data were created using Origin Pro 2017 version 94E (OriginLab Corporation, Northampton, MA, USA).

2.9. Microscopy

Gels prepared as described in section 2.5 were cut into approximately 2 × 2 × 2 mm^3 pieces and fixated overnight in 2.5% glutaraldehyde and 0.1% ruthenium red solution (Langton et al., 2020). The samples were then further fixated in 1% osmium tetraoxide for 2 h, followed by dehydration in a series of ethanol with increasing concentration. For light microscopy (LM), the samples were infiltrated and hardened using Technovit 7100 and sectioned into 1-μm sections using an ultramicrotome (Leica Microsystems GmbH, Leica EM UC6, Wetzlar, Germany). The sections were double-stained with light green and iodine before being examined under a microscope (Nikon, Eclipse Ni–U microscope, Tokyo, Japan) equipped with a 40 × (0.75 NA) apochromatic objective. Images were captured with a Nikon Digital Sight DS-Fi2 camera (Nikon, Tokyo, Japan) with 0.12 μm/pixel.

For scanning electron microscopy, samples were critical point dried (Quorum Technologies Ltd, K850 Critical Point Dryer, East Sussex, UK) after the dehydration step, fractured and sputter-coated with gold (Cressington Scientific Instruments, Sputter coater-108 auto, Watford, UK) before being examined at 5 kV (Hitachi, FlexSEM 1000II, Tokyo, Japan). Images were recorded digitally at a magnification of × 10 000 (9.9 nm/pixel) and × 50 000 (1.98 nm/pixel).

3. Results and discussion

The design used in the study made it possible to investigate the effect of starch and/or fibre on protein gel textural and microstructural properties. It also allowed comparison of the textural properties of gels produced from faba bean protein isolate and gels produced from less pure, hypothetical protein fractions. Dry-fractionated protein concentrate from faba bean flour contains approximately 61% protein, 15% starch and 10% non-starch polysaccharides, while faba bean flour contains approximately 35% protein, 43% starch and 7% non-starch polysaccharides (Saldanha do Carmo et al., 2020). Understanding how the purity of a protein-rich material affects gel textural properties can help in choosing the most suitable source for a certain application.

3.1. Characterisation of raw materials

The chemical composition of the protein, starch and fibre fractions extracted from faba bean cotyledon for use in this study is summarised in Table 1. The cotyledon and hull flours obtained after dehulling of the beans are included for comparison. The protein fraction had a protein content of 77 g/100 g and a relatively high ash content (8 g/100 g). It also contained the highest amount of fat (3.35 g/100 g). The starch fraction contained 86 g starch per 100 g, with 8.1 g of this being resistant starch, and only minor amounts of protein, fibre, fat and ash. The fibre fraction was rich in fibre (28.94 g/100 g NDF and 15.17 g/100 g ADF), but also contained significant amounts of starch (22 g/100 g) and a minor amount of protein. The protein and starch fractions contained higher amounts of protein and starch, respectively, than the cotyledon flour used as the starting material for their extraction. The fibre fraction seemed to contain somewhat higher amounts of cellulose than the cotyledon starting material. However, the cellulose and hemicellulose contents in the fractions and the cotyledon flour were obtained using different methods, and can therefore not be compared directly. Based on literature values, the amounts of NDF and ADF in the fibre fraction were about 50% higher than the amounts normally found in whole (cotyledon plus hull) faba bean flour (Gdala & Buraczewska, 1997). The hull fraction contained less protein and starch, but larger amounts of fibre than the cotyledon flour. This highlights the potential of the hull fraction as a

Table 1
Chemical composition (g/100 g dry weight ± 1 st. dev.) of faba bean cotyledon, faba bean hull and protein, starch and fibre fractions extracted from the cotyledon. N.D. Not determined. N.A. Not applicable. *Expressed as g/100 g sample.

	Fractions extracted from cotyledon			Raw material	
	Protein fraction	Starch fraction	Fibre fraction	Cotyledon	Hull
Crude protein	77.28 ± 0.18	0.48 ± 0.03	5.27 ± 0.12	33.03 ± N. A.	20.38 ± N.A.
Starch	0.27 ± 0.05	86.43 ± 1.39	22.09 ± 0.21	40.31 ± 1.3	19.07 ± 0.63
Resistant starch	N.D.	8.1 ± 0.20	0.40 ± 0.02	N.D.	N.D.
NDF	0.99 ± 0.28	2.19 ± 0.28	28.94 ± 4.37	N.D.	N.D.
ADF	1.32 ± 0.03	1.37 ± 0.14	15.17 ± 0.02	N.D.	N.D.
Lignin	N.D.	N.D.	0.97 ± 0.11	2.97 ± 0.5	8.24 ± 1.07
Cellulose	N.D.	N.D.	14.20 ± 0.09	10.11 ± N. A.	26.88 ± N.A.
Hemicellulose	N.D.	N.D.	13.77 ± 4.34	8.79 ± 0.43	21.25 ± 0.85
Fat	3.35 ± 0.04	0.27 ± 0.03	0.35 ± 0.01	1.6 ± N.A.	1.24 ± N.A.
Ash	8.0 ± 0.72	0.2 ± 0.01	3.5 ± 0.03	3.19 ± 0.21	2.94 ± 0.03
Moisture*	5.3 ± 0.07	7.1 ± 0.01	6.3 ± 0.0	10.87 ± 0.15	10.45 ± 0.16

fibre-rich ingredient in various foods for increased fibre content. It should be noted that a large proportion of the starch and protein in the hull fraction most probably did not originate from the hull itself, but was rather a result of poor separation of the hull and the cotyledon (Frejnagel, Zduńczyk, & Krefft, 1997).

The monosaccharide composition of the fibre fraction, cotyledon and hull flours is shown in Table 2. Due to the presence of starch and cellulose, glucose was the major monosaccharide in all samples. The lower glucose levels in the fibre fraction were the result of starch removal during extraction.

The cotyledon contained higher amounts of arabinose and galactose, but lower amounts of rhamnose, xylose and uronic acids, than the hull. The arabinose and galactose levels in the cotyledon and hull may suggest presence of arabinan and arabinogalactan polysaccharides (Bhatty, 1990). Xylose was not detected in the cotyledon, whereas the hull contained relatively large amounts, suggesting presence of xylans in the hull (Gdala & Buraczewska, 1997). The differences in monosaccharide composition between the hull and the cotyledon indicate that inclusion of the hull during extraction of the fibre fraction could influence the composition of the fibre fraction.

The main difference in monosaccharide composition of the fibre fraction compared with the cotyledon and hull was the high content of arabinose and uronic acids. This suggests relatively high contents of arabinan and/or other arabinose-containing fibres, as well as pectin. Surprisingly, both xylose and rhamnose were detected in the fibre fraction, but not in the cotyledon from which the fraction was extracted. However, weak peaks were identified for both xylose and rhamnose in the cotyledon samples, indicating their presence in low concentrations. Hence, the higher contents in the extracted fibre fraction most likely resulted from up-concentration during extraction.

3.2. Rheology

For the two-component gels, an increase in storage modulus (G') was observed with increased starch and fibre content (Fig. 2a–b). The overall development in G' was similar for all the mixtures, indicating that protein played a dominant role in the gel network (Fig. S1). The loss modulus (G'') followed a similar pattern to G' (results not shown). The tan δ value (G''/G') was in the range 0.13–0.17 for all gels. All values recorded for G', G'' and tan δ of the final gels can be found in Table S2.

The main differences in the development of G' between the samples were the rate of increase in G' during heating and cooling. Both starch and fibre contributed to a higher rate of increase in G' during heating and cooling compared with the gels containing only protein. The G' value of the protein/fibre gels was higher than that of the pure protein gels throughout the whole gelation process. On the other hand, the initial G' value of the starch/protein gels was lower than the initial G' value of the pure protein gels, and overtook it only after reaching a temperature of 75–80 °C (Fig. S2). Thereafter, G' stayed higher for the remainder of the process. Faba bean starch gelatinisation, defined as the peak temperature from differential scanning calorimetry measurements, occurs at a temperature of approximately 70 °C (Ambigaipalan et al., 2011). The pasting temperature of faba bean starch is reported to be around 77 °C (Nilsson et al., 2022). However, one factor that might have influenced the functional properties of the starch used in this study was the alkaline conditions during extraction. Alkaline extraction or alkaline treatment of starch has previously been found to affect the structure, gelatinisation temperature and pasting properties of other starches (Cai et al., 2014; Dokić, Dapčević, Krstonošić, Dokić, & Hadnađev, 2010). Nonetheless, the previously reported functional properties of faba bean starch indicate that the increase in G' with addition of starch to the mixtures was a result of starch swelling, pasting and gelatinisation. The increase during cooling was likely due to starch amylose re-association and network formation. A similar effect has been observed for lentil starch during heating and cooling of pea protein gels (Johansson et al., 2021).

The consistently higher values for the protein/fibre gels compared with the pure protein gels indicates that the increasing effect from the fibre fraction was constant throughout the gelation process. This is consistent with the hydration properties of insoluble sugarcane fibres, which are reported not to change with temperature (Zhuang, Wang, Jiang, Chen, & Zhou, 2020). In general, the samples with increased addition of starch or fibre took longer to equilibrate during the holding time at 25 °C, as seen by the continued increase in G' at the end of the measurement (Fig. 2 and Fig. S1). This could be related to amylose network stabilisation by the starch and water migration due to water adsorption by the starch and/or fibre. However, for many of the starch and fibre containing samples, the increase in G' during the final holding time was not as large as in the 2-component systems (Fig. S1). Further research is needed to confirm and explain the observed increase.

Particles in a gel can be classified as either active or inactive fillers, depending on their interaction with the gel matrix (Dille, Draget, & Hattrem, 2015). Both modified cassava starch and insoluble dietary fibre from sugarcane have previously been shown to create thermodynamically incompatible and phase-separated gels with myofibrillar protein (Zhuang, Jiang, et al., 2020). Similar observations have been made for corn starch and soy protein gels (Li, Yeh, & Fan, 2007). Further studies of the investigated faba bean systems would be needed to specify the interaction between the starch/fibre surface and the protein matrix to elucidate whether they act as active or inactive filler particles. A decrease in G' has previously been correlated with addition of inactive filler particles and starch to protein gels (Dille et al., 2015; Yu, Ren, Zhao, Cui, & Liu, 2020). Despite this, we observed an increase in G' for the mixed gels compared with the pure protein gels. An increase in G' after heating of soy milk has been observed previously following inclusion of okara fibre with varying particle sizes (Lan et al., 2020). One possible explanation for the increase in G' with addition of fibre is water adsorption and improved moisture stability. This has been reported for myofibrillar protein gels with insoluble fibres from sugarcane (Zhuang, Wang, et al., 2020). Another potential explanation for the increase in G' could be that the starch granules and fibre particles act as active fillers. Active filler particles can be expected to reinforce the gel and increase the overall shear modulus if the filler particle has a higher modulus value than the surrounding gel matrix (Dille et al., 2015).

Contour plots at different temperatures visualised the differences in G' between samples during gelation (Fig. 2I–V). In contour plots at additional temperatures (data not shown), it was observed that a high proportion of the fibre fraction resulted in the highest G' values until the mixtures reached about 80 °C. Above that temperature, high starch content also resulted in comparably high G' and the highest values were observed when the proportions of fibre and starch were both high. During cooling, a high proportion of starch contributed more to the increase in G' than addition of fibre. This was apparent as a shift in the high-value region (shown in red in Fig. 2I–V) from the high fibre/high starch region towards the high starch region. The slight decrease in G' observed at high starch and high fibre, compared with only high starch or only high fibre, could be due to the large number of particles disrupting the continuous protein phase.

Table 2
Relative percentage of measured monosaccharides in the fibre fraction, the faba bean starting material (cotyledon) and hull. N.D. Not detected. N.Q. Detected but not quantified due to peaks being too vague for accurate quantification.

	Extracted fraction	Raw material	
	Fibre fraction	Cotyledon	Hull
Arabinose	20.39 ± 0.07	4.69 ± 0.01	3.22 ± 0.04
Rhamnose	2.34 ± 0.06	N.Q.	0.68 ± 0.02
Galactose	5.23 ± 0.04	4.70 ± 0.01	3.31 ± 0.02
Glucose	55.02 ± 0.03	88.70 ± 0.31	77.60 ± 0.71
Xylose	4.22 ± 0.02	N.Q.	7.79 ± 0.08
Mannose	N.D.	N.D.	N.D.
Uronic acids	12.80 ± 0.11	1.91 ± 0.33	7.40 ± 0.87

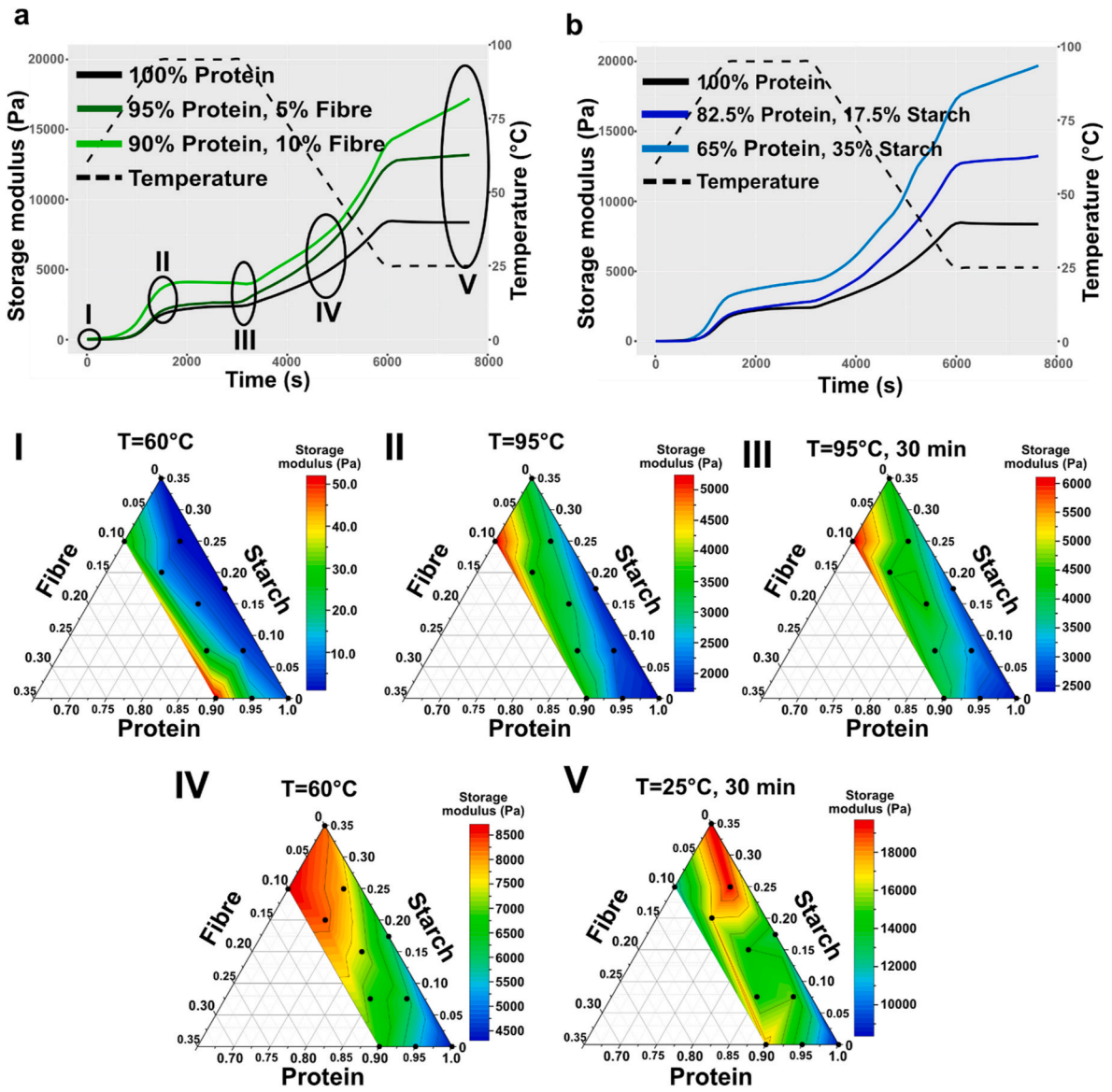

Fig. 2. Storage modulus (G′) throughout the gelation process of (a) protein/fibre gels and (b) protein/starch gels. Percentages of protein, starch and fibre in (a) and (b) are relative to the total amount of flour added. Panels I–V are contour plots of the storage modulus of all gels in the design at different time points during the gelation process, as also indicated in Fig. 2a. The black dots correspond to the measuring points. Axes correspond to proportion of protein/starch/fibre-fraction in total flour added.

3.3. Compression tests

The fracture stress, fracture strain and Young's modulus of gels prepared with different ratios of the extracted protein, starch and fibre fractions are visualised as contour plots in Fig. 3. The exact values with standard deviations are supplied in Table S3. The fracture stress of the gels increased with an increasing proportion of protein. The lowest gel strength was observed at a low protein content in combination with high fibre and high starch content. The fracture strain showed similar behaviour, with an increase with increasing protein content, but the lowest values were obtained for gels containing low amounts of protein and fibre combined with high amounts of starch. The lowest Young's modulus values were observed for gels high in protein and the highest values were obtained for gels with high starch content and/or high fibre content.

A similar decrease in hardness has been observed previously in texture profile analysis (TPA) of soy protein gels with an increased proportion of added native corn starch (Yu et al., 2020). This decrease in hardness was explained in that study by water adsorption by the starch, suppressing the protein aggregation during gelation, and leaked amylose disrupting the protein network formation. At higher starch concentrations, those authors also observed that starch induced formation of discontinuous gels, decreasing the TPA parameters. A reduction in the hardness of soy protein tofu after addition of okara dietary fibre with varying particle size has also been reported (Ullah et al., 2019; Wei et al., 2018). This decline in hardness of tofu with added IDF occurred despite an increase in water entrapment, which on its own could be expected to increase the strength of the gels. Instead, it was suggested that the addition of IDF reduced textural properties by creating a more inhomogeneous and unstable network structure. Large particles have also been suggested to explain the reduction in hardness of tofu gels by unfavourably affecting chain association during gelation, reducing the structural stability (Liu, Chien, & Kuo, 2013).

The larger fibre particles and gelatinised starch granules in our mixed gels most likely acted as inactive filler particles. A previous study examining gellan gum gels and the effect of adding gel particles

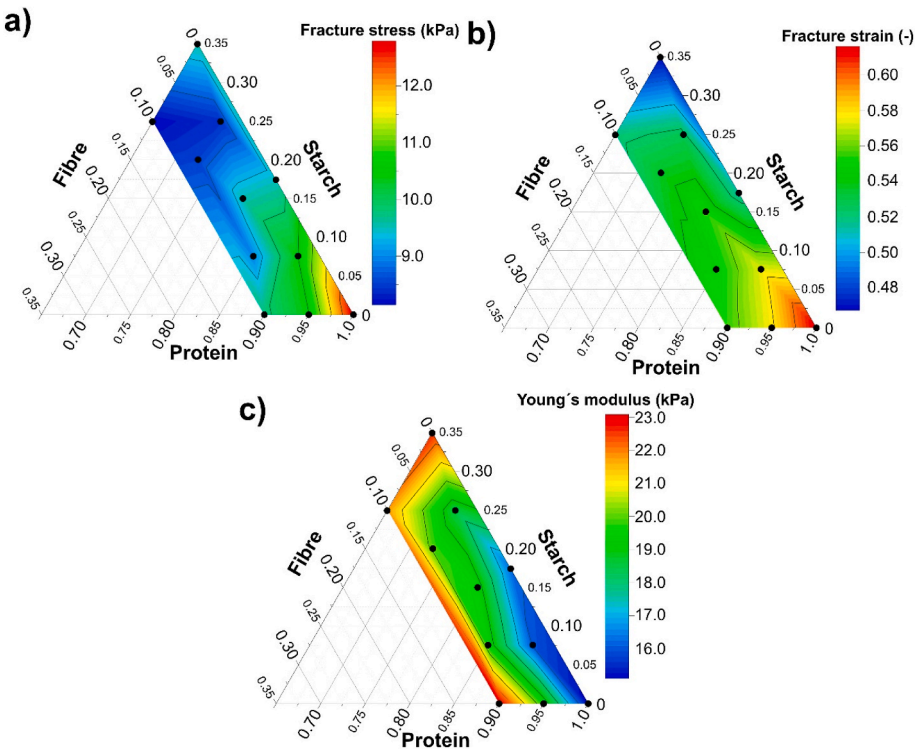

Fig. 3. Contour plots of (a) fracture stress, (b) fracture strain and (c) Young's modulus for gels prepared with different ratios of extracted protein, starch and fibre fractions. The black dots correspond to the measuring points. Axes correspond to proportion of protein/starch/fibre-fraction in total flour added.

approximately 100 μm in size found that addition of gel particles reduced the fracture stress and fracture strain of the gels, where the particles were assumed to act as inactive fillers (Moritaka, Takeuchi, Okoshi, & Fukuba, 2002). An earlier study of whey protein emulsion gels showed the importance of the interaction between filler particles and the gel matrix, with a decrease in gel strength with addition of inactive filler particles and an increase with addition of active filler particles (Dickinson & Chen, 1999).

3.4. Regression analysis of compression test and rheology

The results of regression analysis of the compression test and rheology results are shown in Table 3. Nonlinear blending effects were included in the models for all responses (fracture stress, fracture strain, Young's modulus and G'). However, it should be noted that including nonlinear blending terms in reduced models for mixture designs does not necessarily mean that the components included in these terms (e.g. Protein*Starch for fracture stress) are those chemically causing the nonlinear blending behaviour as a function of composition (Redgate et al., 1992). An additional reason for this is multicollinearity among linear and nonlinear terms (Redgate et al., 1992), where one of the components in the mixture can be written as a function of the others due to the constraint that their proportions should always add up to one.

The relatively high values of protein coefficient obtained for the fracture properties indicate that protein content was the main determining factor for fracture stress and fracture strain. On the other hand, the starch and fibre coefficients were higher for Young's modulus and G'. For G', the interaction term Starch*Fibre had the highest coefficient, indicating a strong interaction.

All models were statistically significant ($p < 0.05$) and did not exhibit any lack-of-fit as indicated by p-values above 0.05. Fracture stress, fracture strain and Young's modulus showed relatively high $R^2(adj)$ and low root mean square error (RMSE) in relation to the overall mean response. This indicates that the model fitted the data well and

Table 3
Regression coefficient (β_i) and R^2 for models fitted to the equation $Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} \times 1 \times_2 + \beta_{13} \times 1 \times_3 + \beta_{23} \times 2 \times_3 + \beta_{123} X_1 X_2 X_3$, where Y is the studied response and X_1, X_2 and X_3 are the proportions of protein, starch and fibre fractions, respectively. Forward selection and minimising of the AIC was used to remove insignificant model terms. Values within bracket are standard error of the regression coefficients. RMSE (root mean square error) is the standard deviation of the residuals.

	Fracture stress		Fracture strain		Young's modulus		Final G'	
	Coefficient (Std.Error)	p-value	Coefficient (Std.Error)	p-value	Coefficient (Std.Error)	p-value	Coefficient (Std.Error)	p-value
(Protein-0.65)/0.35	12.18 (0.38)	<0.0001	0.61 (0.01)	<0.0001	15.0 (0.8)	<0.0001	9300 (1300)	<0.0001
Starch/0.35	9.50 (0.44)	<0.0001	0.47 (0.01)	<0.0001	22.3 (0.8)	<0.0001	20900 (1400)	<0.0001
Fibre/0.35	4.24 (1.29)	<0.0001	0.41 (0.06)	<0.0001	43.9 (3.5)	<0.0001	37400 (6500)	<0.0001
Protein*Starch	−3.51 (1.56)	0.0445	–	–	−7.3 (2.7)	0.0210	–	–
Protein*Fibre	–	–	–	–	−32.2 (7.3)	0.0010	–	–
Starch*Fibre	–	–	0.39 (0.12)	0.0055	–	–	−58300 (13600)	0.0004
Protein*Starch*Fibre	–	–	–	–	–	–	–	–
Lack of fit (p-value)	0.26		0.57		0.33		0.12	
RMSE	0.52		0.014		0.89		2360	
R^2 (adjusted)	77.5%		81.0%		84.0%		56.4%	
p-value (model)	<0.0001		<0.0001		<0.0001		0.0004	

could be useful for understanding behaviour tendencies in the region studied. Lower $R^2(adj)$ combined with high RMSE limited the usefulness of the model for the final G'. This poor fit of the model for the final G' was partly a result of the relatively large variation between replicates. Ternary plots of the final models are shown in Fig. S3.

3.5. Microstructure

The microstructure of the gels at the four vertex points, the centre point and an additional point on the starch-protein axis was evaluated by LM and SEM (Figs. 4 and 5). For all gels, LM revealed a continuous protein phase with homogeneous microstructure at the observed level of magnification (Fig. 4). Dense homogeneous protein microstructure has previously been observed for faba bean protein gels at pH 7 (Langton et al., 2020). The starch was present as swollen and deformed starch granules, as well as what were assumed to be small amylose aggregates from material leaked from the granules. Cell wall fragments were evident as white/non-stained parts throughout the gels including the fibre fraction (Fig. 4). Aggregation of amylose was observed on the surface of starch granules and, to a smaller extent, on the surface of larger fibre particles. Phase separation between the starch and protein with starch aggregated on surfaces and in smaller aggregates throughout the protein matrix has been reported previously in mixed protein/starch gels (Johansson et al., 2021; Li & Yeh, 2003).

The amylose aggregates observed by LM were also visible throughout the protein network in the SEM micrographs of gels containing starch (Fig. 5a). The amylose was present as aggregates inside spherical cavities (approximately 1 μm in diameter). Similar cavities were observed in the protein/fibre gels with what was assumed to be smaller fibre particles. No or few such cavities were observed in the protein gels. As observed by LM, SEM revealed amylose aggregates and amylose networks on the surface and in close proximity to starch granules and some of the fibre particles (micrographs not shown).

No major differences in the gel protein network were observed in the SEM micrographs after addition of fibre and/or starch fraction compared with the pure protein gel, despite differences in protein concentration (Fig. 5b). This could be a result of water adsorption by the starch and the fibre, which would increase the effective protein concentration in the continuous phase and keep it relatively constant for the different gels. Others have shown that starch and amylopectin can affect protein networks formed during heat-induced gelation (Olsson et al., 2002; Shim & Mulvaney, 2001). An additional reason for the lack of differences could be the high content of solid material in our gels, limiting diffusion of the leaked starch amylose. A previous study observed smaller differences in microstructure between starch/protein gels prepared at different pH and temperatures as the solids content was increased from 15% to 30% (Shim & Mulvaney, 2001). The high solids content in our gels and protein gelation, as indicated by a drastic increase in G', starting at a temperature close to the faba bean starch gelatinisation temperature, could both have limited the ability of amylose to form a continuous network.

3.6. Texture and microstructure

The general development in G' was similar for all gels, suggesting that protein was the dominant component during gelation. This is in agreement with protein forming the continuous phase for all gels, as observed by LM, and the protein network structure being very similar for all the gels, as observed by SEM.

Interactions between protein, starch and fibre in mixed gels influenced the textural properties and microstructure. Most heat-induced mixed protein-polysaccharide gels are obtained from thermal

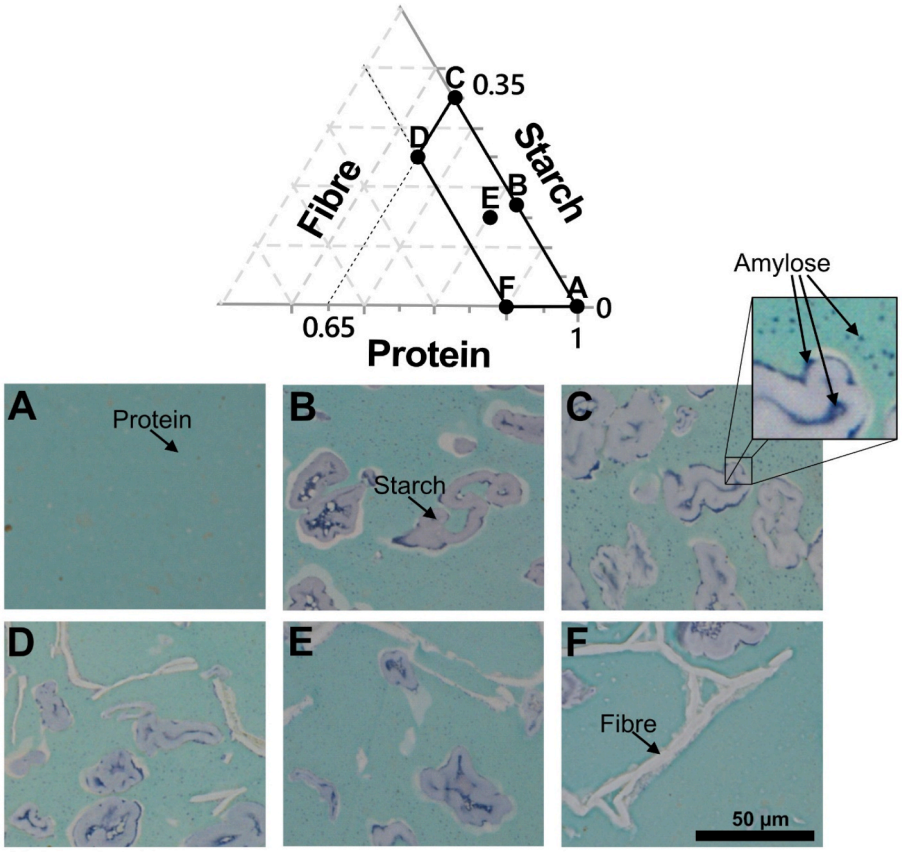

Fig. 4. Light micrographs of gels with different ratios of the extracted fractions (A–F) as indicated by the design points (filled circles) in the mixture triangle. Magnification: × 40. Pixels: 2560 × 1920.

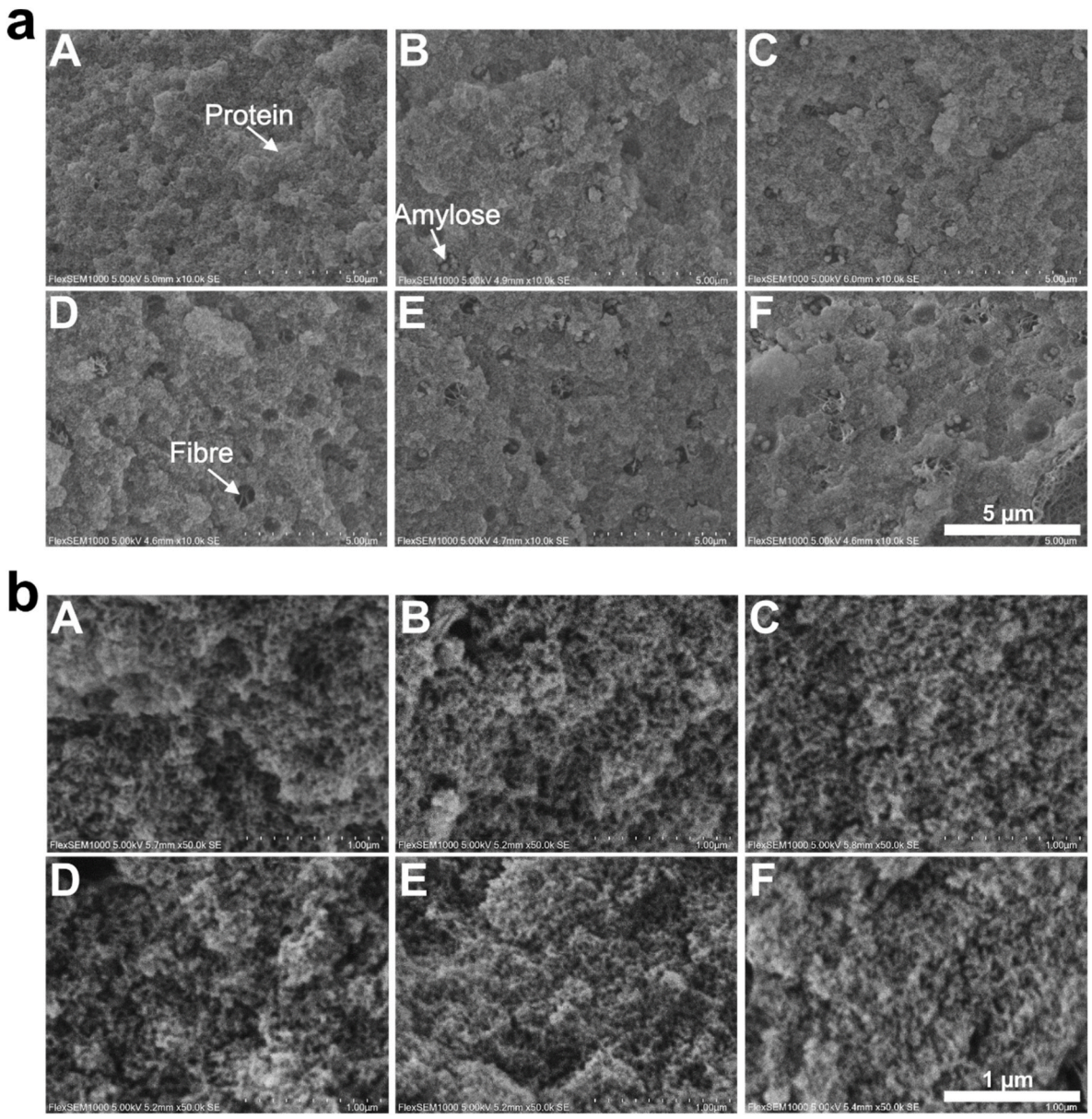

Fig. 5. Scanning electron micrographs of gels with different ratios of the extracted fractions corresponding to the design points marked in the mixture triangle seen in Fig. 4. Magnification: × 10.000 (a), × 50.000 (b). Pixels: 1280 × 960 pixels.

treatment under thermodynamic incompatibility conditions (Schmitt, Sanchez, Desobry-Banon, & Hardy, 1998). Starch and fibre have previously been identified as non-interacting or inactive fillers in other protein gels (Li et al., 2007; Zhuang, Jiang, et al., 2020). Addition of starch and fibre will increase the heterogeneity of the gel microstructure and affect the textural properties. However, the interaction between starch/fibre and protein depends on multiple factors such as source, surface properties, chemical modifications and the presence of other components. Interactions have been reported in e.g. wheat dough systems and fibre particles with modified surfaces (Ryan & Brewer, 2005; Wei et al., 2018).

Fracture of a gel is suggested to occur by propagation of cracks (Dille et al., 2015). Cracks are often formed at structural defects, such as filler particles, acting as stress concentrators during deformation (Dille et al., 2015). Similarly, microscale inhomogeneity based on apparent coarseness of the protein network has previously been correlated with lower fracture stress in pea protein gels (Munialo et al., 2014). The porous nature of the gel could cause step-wise crack growth, resulting in lower fracture stress (Munialo et al., 2014). Hence, inhomogeneities in the form of amylose/fibre-filled cavities, starch granules and larger fibre particles might explain the reduced fracture stress and fracture strain after addition of starch and/or fibre in this study.

While addition of fibre and starch fraction resulted in a decrease in fracture stress and fracture strain, an increase in Young's modulus and G' was observed. A similar increase in G' of heated soy milk after inclusion of large fibre particles (250–380 μm), accompanied by a decrease in textural properties of tofu prepared from the same raw materials, has been observed by others (Lan et al., 2020). Large deformation properties, such as fracture stress and fracture strain, are affected by inhomogeneities in the matrix. On the other hand, small deformation properties, such as Young's modulus and G', could be expected to be less dependent on inhomogeneity and inactive filler particles, and more dependent on the continuous phase. However, filler particles will also affect small deformation properties, especially active filler particles (Dille et al., 2015). Hence, the larger fibre particles, starch granules and small amylose/fibre aggregates throughout the protein matrix could explain the reduction in fracture stress and fracture strain seen in this study, while addition of fibre/starch could have increased G' through

water adsorption and moisture stability. The latter could have increased the effective protein concentration in the surrounding protein matrix and potentially overshadowed the negative effect of starch and fibre on G' and Young's modulus as filler particles.

Differences in heating and cooling rates for the rheology tests and gels prepared for compression tests and microscopy will influence the gelation and affect the comparability of the results from these measurements (Pelgrom, Boom, & Schutyser, 2015; Sun & Arntfield, 2011). The heating and cooling rates of gels prepared for compression and microscopy in this study exceeded the rates used during the rheology measurements by a factor of 10 or more. Hence, the lower heating rate during rheology measurements might have allowed starch gelatinisation to progress further before protein gelation occurred compared with the gels prepared for compression and microscopy. Heating and cooling rates have previously been shown to affect the gelatinisation of certain starches, with e.g. the pasting properties of wheat and semolina flour being affected by heating/cooling rate and those of rice flour being less affected (Mariotti, Zardi, Lucisano, & Pagani, 2005). For wheat and semolina flour, higher rates have been found to result in higher peak and end viscosity during viscoamylographic tests (Mariotti et al., 2005). A similar increase in viscosity at higher heating/cooling rates has been observed for faba bean starch, and can potentially be linked to more pronounced granular swelling at higher heating rates (Nilsson et al., 2022).

The cooling rate also significantly affects protein gelation, with a lower rate increasing gel strength (Sun & Arntfield, 2011). In mixed systems, slower cooling rates will give more time for the protein to arrange in a network, whereas fast heating rates can reduce phase separation (Pelgrom et al., 2015). It has been shown that the contribution from starch and the properties of the gel and gel network of starch/-protein mixed gels will differ depending on which component gels first (Muhrbeck & Eliasson, 1991). Those authors found that when the starch network forms before the protein network, the protein and starch supplement each other and form two continuous networks without any specific interaction. On the other hand, if the protein gels before the starch, this hinders diffusion and aggregation of starch amylose and the starch will not form a continuous network and will instead act as a filler material (Muhrbeck & Eliasson, 1991). Considering this, the slower heating rate in the rheometer in the present study gave more time for the amylose to leak into solution and aggregate compared with the fast heating during gel formation in the water bath. Hence, the starch might have had a larger positive effect on the G' value, compared with the effect on the fracture properties. The high solids content might also have limited amylose network formation due to reduced diffusion and steric hindrance.

4. Conclusions

Substituting protein for fibre and/or starch reduced gel fracture stress and fracture strain, but increased Young's modulus and storage modulus. Light microscopy revealed that protein formed the continuous phase for all mixtures studied (protein 65–100%, starch 0–35%, fibre 0–10%). Starch granules and fibre particles were distributed throughout the gels. Scanning electron micrographs indicated no clear differences between the protein matrix of the different gels, but revealed small cavities (<1 μm) in the protein matrix where fibre and amylose seemed to have aggregated. The reduction in fracture stress and fracture strain with addition of fibre and/or starch could be the result of inhomogeneities created in the protein matrix. The increase in Young's modulus and storage modulus was possibly more dependent on the continuous phase, where water adsorption by fibre and starch granules increased the protein concentration and moisture stability in the surrounding matrix, or possibly by starch granules and fibre particles acting as active fillers reinforcing the gel matrix.

In a broader perspective, our results indicate that the purity of the faba bean protein fraction can significantly affect the final textural properties of the gel. With a constant solids content, we observed significant differences in the textural properties of gels produced from a protein isolate compared with gels produced from a mixture of protein, starch and fibre with ratios more similar to those in a dry-fractionated protein concentrate. Differences in fracture properties and storage modulus were observed even as a small amount of protein was replaced with starch and/or fibre fraction. This highlights the possible usefulness of wet-fractionated protein isolates despite their higher environmental impact compared with dry-fractionated protein concentrates.

Credit authorship contribution statement

Mathias Johansson: Conceptualization; Formal analysis (statistics, data analysis); Investigation; Methodology; Validation; Visualization; Writing - original draft; Writing - review & editing. **Daniel Johansson:** Conceptualization; Methodology; **Anna Ström:** Writing - review & editing. **Jesper Rydén:** Formal analysis (statistics); Writing - review & editing. **Klara Nilsson:** Investigation; Writing - review & editing. **Jakob Karlsson:** Investigation. **Rosana Moriana**: Investigation; Writing - review & editing. **Maud Langton:** Conceptualization; Funding acquisition; Methodology; Supervision; Writing - review & editing.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodhyd.2022.107741.

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