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Extrusion-aided Faba Bean Protein Fractionation

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Extrusion-aided Faba Bean Protein Fractionation

MB Devi Marhendraswari

Master's Thesis
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Title of thesis Extrusion-aided Faba Bean Protein Fractionation

Programme Biological and Chemical Engineering for a Sustainable Bio-economy

Thesis supervisor Prof. Paula Jouhten

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Abstract

Sustaining the availability of plant-based protein alternatives to meet the growing demand is crucial. Texturized vegetable protein (TVP) is one of the plant-based alternatives available; however, the production of TVP involves energy and water-intensive processes for protein enrichment of the raw material. The state-of-the-art dry-extrusion-aided plant protein fractionation process, recently patented by VTT, presents a promising alternative for the existing protein-enrichment methods. However, despite of its potential, there is limited knowledge regarding the impact of extrusion conditions on the properties of the produced fractions. Thus, this thesis aims at identifying extrusion conditions favouring starch-protein separation, focusing on the utilization of the untapped potential of Faba bean (*Vicia faba*), a locally cultivated legume known for its high protein content.

Through process optimization, different conditions favouring high protein content and high protein yields in the protein-rich fraction were identified. The optimized conditions for protein content yielded a protein content of 76.78% with a protein yield of 59.84%. Additionally, when the focus shifted towards maximizing protein yield, a protein yield of 84.85% with a corresponding protein content of 71.02% was obtained. Notably, these results were relatively higher than protein concentrate and TVP available in the market.

Moreover, the optimized conditions not only resulted in high protein content and yield of protein-rich extrudates, but also demonstrated comparable techno-functional properties to commercial TVP. Additionally, the extent of protein texturization during the extrusion process determined the techno-functional attributes of the protein-rich fraction.

The dry extrusion-aided fractionation process is expected to be a more sustainable approach compared to conventional TVP production by bypassing resource-intensive flour fractionation. This research contributes valuable insights into facilitating the development of innovative and more sustainable plant-based protein alternatives.

Keywords Plant-based protein; Faba bean; Texturized-vegetable proteins

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Abstract (Estonian)

Kasvava nõudluse rahuldamiseks on väga oluline tagada taimse valgu alternatiivide kättesaadavus. Tekstureeritud taimne valk (TVP) on üks olemasolevatest taimsetest alternatiividest, kuid TVP tootmine hõlmab energia- ja veemahukaid protsesse tooraine valgu rikastamiseks. VTT poolt hiljuti patenteeritud kaasaegne kuivekstrusiooni abil toimuv taimse valgu fraktsioneerimise protsess on paljulubav alternatiiv olemasolevatele valgu rikastamise meetoditele. Vaatamata selle potentsiaalile on siiski piiratud teadmised ekstrusioonitingimuste mõju kohta toodetud fraktsioonide omadustele. Seega on käesoleva väitekirja eesmärk kindlaks teha tärklise ja valkude eraldamist soodustavad ekstrusioonitingimused, keskendudes Faba oa (Vicia faba), kohalikult kasvatatud ja kõrge valgusisalduse poolest tuntud kaunvilja, seni kasutamata potentsiaali ärakasutamisele.

Protsessi optimeerimise abil määrati kindlaks erinevad tingimused, mis soodustavad suurt valgusisaldust ja suurt valgurikka fraktsiooni valgusaagist. Valgusisalduse suhtes optimeeritud tingimused andsid 76,78 % valgusisalduse ja 59,84 % valgusaagise. Lisaks sellele, kui keskenduti valgusaagise maksimeerimisele, saadi valgusaagis 84,85 % ja vastav valgusisaldus 71,02 %. Need tulemused olid suhteliselt kõrgemad kui turul saadaolevad valgukontsentraadid ja TVP.

Lisaks sellele ei andnud optimeeritud tingimused mitte ainult kõrget valgusisaldust ja valgurikka ekstrudraadi saagist, vaid näitasid ka võrreldavaid tehnilis-funktsionaalseid omadusi kaubandusliku TVPga. Lisaks sellele määras valgurikka fraktsiooni tehnofunktsionaalsed omadused kindlaks valgu tekstureerimise ulatus ekstrusiooniprotsessi käigus.

Eeldatakse, et kuiv ekstrusiooni abil toimuv fraktsioneerimisprotsess on tavapärase TVP tootmisega võrreldes jätkusuutlikum lähenemisviis, kuna sellega väldi-takse ressursimahukat jahu fraktsioneerimist. See uuring annab väärtusliku panuse uuenduslike ja jätkusuutlikumate taimse valgu alternatiivide väljatöötamise hõlbustamisse.

Keywords Taimne valk; Faba oad; Tekstuuritud taimne valk.

AUTHOR'S DECLARATION

Hereby I declare, that I have written this thesis independently.
No academic degree has been applied for based on this material. All works, major viewpoints and data of the other authors used in this thesis have been referenced.

14/06/2023

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TalTech Department`s title

THESIS TASK

Student: MB Devi Marhendraswari, 220095EV
Study programme, (KEVM) European Master in Biological and Chemical Engineering for a Sustainable Bio-economy
main speciality:Food Technology
Supervisor(s): Professor, Petri-Jaan Lahtvee
Professor, Paula Jouhten
Consultants: Dr. Outi Mattila, Senior Research Scientist
VTT Technical Research Centre of Finland

Thesis topic:
(in English) Optimization of extrusion conditions favouring protein-starch separation of Faba bean and techno-functional evaluation of the resulting fractions
(in Estonian) Faba oa valgu-tärklise eraldamist soodustavate ekstrusioonitingimuste optimeerimine ja saadud fraktsioonide tehnilis-funktsionaalne hindamine

Thesis main objectives:

1. Identifying extrusion conditions favouring separation of starch-rich fraction and protein-rich fraction of Faba bean focusing on protein content and protein yield
2. Evaluating the techno-functional analysis of the starch-rich fraction and protein-rich fraction of fractionated Faba bean

Thesis tasks and time schedule:

No	Task description	Deadline
1.	Literature review	1.5 month
2.	Pre-trials on extrusion conditions for determining the limit of variable in the extrusion conditions, determining the protein content, and establishing a suitable method for controlling feed rate	1 month
3.	Extrusion work based on the experimental design and analysis of the obtained data	1 month
4.	Extrusion work at optimized conditions	2 weeks
5.	Technofunctional analysis of fractions produced from extrusion at optimized conditions	2 weeks
6.	Writing thesis report	2 weeks

Language: English **Deadline for submission of thesis:** 15/06/23

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Preface

I would like to thank Professor Paula Jouhten, Professor Nesli Sözer, and my instructors Dr Outi Mattila for their guidance.

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I also thank myself for surviving this roller-coaster journey and my partner who keeps buying me nice food even though we are 13,118km away.

Lastly, greater thanks to my parents who always keep me in their prayers.

Otaniemi, 15 June 2023

MB Devi Marhendraswari

Symbols and abbreviations

Abbreviations

L/V	legumins/vicilins
TVP	texturized vegetable proteins
SME	Specific mechanical energy
RT	room temperature
WAC	water absorption capacity
OAC	oil absorption capacity
RHC	rehydration capacity
BW	boiling water
RTW	room temperature water

1 Introduction

Ensuring the availability of protein to sustain the growing global population is crucial. However, given the adverse effects of the extensive meat consumption to the environment (15% of global greenhouse gases emissions) (Willett et al., 2019), it is crucial to switch from animal-based protein to more sustainable protein source. Having two-third less carbon footprint than meat, plant-based protein is one of the more sustainable protein alternatives (Detzel et al., 2022). Additionally, adopting a plant-based diet has been associated to a reduced prevalence of chronic diseases and positive changes in gut microbiome composition, leading to reduced mortality (Ferrocino et al., 2015; Orlich et al., 2013; Tantamango-Bartley et al., 2013).

Many advantages can be gained by shifting diet to its plant-based alternatives. However, concerns arise when relying on imported materials, impacting continuous production, and causing environmental harm through transportation. Hence, the use of plant sources that can be cultivated locally will bring major benefits for securing its availability and lessening negative impacts to the environment.

Legumes are good candidates for protein alternative sources as they have moderately to highly digestible proteins (Semba et al., 2021). Moreover, pulses (dried-edible seeds of legumes) are rich in lysine, leucine, aspartic acid, glutamic acid, and arginine. However, they lack of sulfur-containing amino acids. Hence, it is essential to incorporate cereals and other amino acids containing sulfur, as well as tryptophan-rich foods, into the diet alongside pulses to achieve a well-balanced nutritional intake (Boye et al., 2010). Among the other legumes, faba beans (*Vicia faba*) is an excellent yet untapped source of plant protein; not only because it has high protein content, but also because of its agronomic advantages.

One of the plant-based protein alternatives available in the market is texturized vegetable protein (TVP). TVP is plant-based food ingredient made of protein-enriched soy flour or other plant source alternatives such as peas and lentils. TVP is produced by low-moisture extrusion process, resulting in dry texturized protein crumbles having meat-like texture and appearance.

In the current market, TVP production involves the utilization of protein-concentrated flour obtained through energy-intensive fractionation techniques from legume and grain flour. These techniques can be categorized into two types: dry and wet fractionation. Dry fractionation is generally considered as a greener option because it does not involve the use of water and chemicals. However, the protein content of the product is typically lower in comparison

to wet fractionation (Augustin & Cole, 2022; Martinez et al., 2016; Vogelsang-O'Dwyer et al., 2020).

A cutting-edge, dry extrusion-aided plant protein fractionation process has recently been patented by VTT. The extrusion process yields a mixture of two types of extrudates: protein-rich fraction and starch-rich fraction. Protein-rich fraction produced from this process is texturized, making it comparable with conventional TVP in the market. Moreover, in comparison to conventional TVP production, this novel process presents a potentially more sustainable approach by bypassing the resource-intensive flour fractionation process required for the protein-enriched raw material used.

Segregative phase separation, occurring within specific conditions, is hypothesized to be the undelaying mechanism responsible for the separation of two biopolymers—protein and polysaccharides. However, a comprehensive understanding of the conditions that facilitate segregative phase separation in extrusion-aided plant protein fractionation is currently lacking. Given the potential of this process in promoting more sustainable plant-based protein alternative ingredients, this thesis aims at identifying extrusion conditions favouring separation, with a focus on optimizing protein content of the protein-rich fraction and protein yield and evaluate the techno-functional properties of the resulting fractions. Additionally, the scientific mechanism involved for biopolymers separation in extrusion-aided plant protein fractionation could be better understood through the findings from the optimization of extrusion process and techno-functional analysis.

2 Literature review

2.1 Faba beans

Faba bean is considered as high protein pulse, having protein content of around 27%, higher than most pulses and peas in the market, such as chickpeas (19.53%), beans (22.17%), and lentils (22.15%) (Chávez-Murillo et al., 2018). Moreover, apart from its superior nutrients, faba bean also possess agronomic advantages. Faba bean has a good capability in nitrogen fixation which can improve soil quality (Herridge et al., 2008), ability to be grown easily for crop rotation and good adaptability to diverse climates including in boreal climate (Lizarazo et al., 2015), as well as its capability to sequester carbon, thus improving soil health and reducing greenhouse gases (Augustin & Cole, 2022).

While the faba bean has a lower protein content compared to soy (40%), it is important to note that most faba bean varieties available in the market are non-genetically modified (non-GMO). Additionally, faba bean is not classified as a regulated allergen, making it practicable to be marketed in the EU countries (Redondo-Cuenca et al., 2007; Wunderlich & Gatto, 2015). Furthermore, as it is reported by Çalışkantürk Karataş et al., (2017), nutritional benefits of faba beans include its high content of mineral and bioactive compounds including dietary fibers as well as phenolic compounds. Therefore, faba bean is not only a more sustainable source of plant-based protein, but also offering health-enhancing potential.

Similar to most pulses, globulin is the most abundant protein type in faba bean. It is classified based on its sedimentation into two categories: legumins and vicilins. Legumins are hexameric globulins possessing acidic (α) and basic (β) peptide side chain. These chains are connected by disulfide bond (Müntz, 1998). On the other hand, vicilins are globulin proteins having trimeric structure composed of different subunits; these subunits are glycosylated and contain no cysteine, therefore they are unable to form disulfide bonds (Utsumi, 1992). Legumins to vicilins (L/V) ratio in faba bean protein characterization is one of the influential factors determining the physical and functional properties of faba bean (Martinez et al., 2016).

On the other hand, faba bean contains anti-nutritional factors, including tannins, lectins, phytic acids and digestive enzyme inhibitors which can induce toxic effects and reduce protein digestibility (Mattila et al., 2018). A particular anti-nutritional compound in faba bean posing negative health effect is pyrimidine glycosides (vicine and convicine). The amount of vicine and convicine in 16 faba bean varieties was reported by Ivarsson & Neil, (2018); the

value ranged from 2.48 to 4.41 g/kg for vicine and convicine, respectively. The presence of vicine and convicine in diet cause favism. Favism refers to a hereditary hemolytic anemia that occurs in individuals who who deficit in glucose-6-phosphate dehydrogenase (G6PD). This condition leads to severe hemolysis following the consumption of faba bean (Luzzatto & Arese, 2018).

Despite the concerns regarding its health effects to some individuals, considering the potential of faba bean as nutritious and sustainable plant protein, some research explored the applicability of faba bean to be incorporated into food products. Vogelsang-O'Dwyer et al., (2020) compared the techno-functional, nutritional, and environmental efficacy of dry and wet fractionated faba bean protein. As compared to the solvent extraction (wet fractionation), the solubility and foaming capacity of dry fractionated protein was significantly greater. This is because the denaturation of protein during the extraction and drying increase the hydrophobicity of the protein, thus lowering foaming capacity. On the other hand, wet fractionated protein showed a substantially enhanced protein digestibility throughout digestion process as compared to the dry-fractionated one (Vogelsang-O'Dwyer et al., 2020).

Additionally, protein extracted using wet fractionation showed no detection of vicine and convicine. However, the dry-fractionated protein concentrate had similar content of vicine and convicine with dehulled faba bean flour. This finding suggests that vicine and convicine are evenly dispersed within the cotyledon and are not concentrated with the protein during milling of the raw materials (Vogelsang-O'Dwyer et al., 2020). As regards to the FODMAP (fermentable oligo-, di- and monosaccharides and polyols) which cause disturbing intestinal symptoms to many people, wet extraction process eliminated most of the galacto-oligosaccharides while dry fractionation slightly increased their concentration. As for the environmental assessment, dry fractionation is reported to have less environmental impacts than that of wet extraction (Vogelsang-O'Dwyer et al., 2020).

Table 1. Protein and amino acids composition of faba bean flour (Martineau-Côté et al., 2022; Świątkiewicz et al., 2018)

Protein type	**Faba bean** (in g/100g protein)
Globulins:	69-78.1
Glutelin:	12-18.4
Prolamins:	1.83-3.57
Albumins:	1.41-3.01
Amino acid composition	
Aspartic acid	10.74
Threonine	3.4
Serine	4.67
Glutamic acid	16.51
Proline	3.94
Glycine	4.73
Alanine	4.15
Cysteine	1.33
Valine	4.31
Methionine	0.87
Isoleucine	3.94
Leucine	7.47
Tyrosine	2.78
Phenylalanine	4.19
Lysine	7.08
Histidine	2.41
Arginine	9.46

2.2 Texturized Vegetable Protein

Texturized Vegetable Protein (TVP) are manufactured from edible sources such as pulses, soy, and cereals (Lyu et al., 2022). Typically, it is prepared from flour that has been enriched in protein content. The definition of TVP according to Featherstone, (2015) is a 'food products that have been transformed from a flour-type material into new material having meat-like texture'. TVP is distinguished by its structural integrity and texture that can resist hydration and other procedures in the food processing and it has been used as a meat extender, with some advantages including maintaining the nutritional value of food products while reducing overall expenses (Penfield & Campbell, 1990)

Extrusion is a widely used commercial technology for produce TVP production. The main aim of the extrusion process in the TVP production is to texturize the amorphous plant protein by denaturation of the protein. According to Featherstone, (2015), during the extrusion process, flour, water, sodium chloride, and additional ingredients is thoroughly mixed. This mixture is then subjected to a high pressure within a cooker extruder and undergoes expansion once it exits the extruder die. The precise dimensions and configuration of the extruded material are governed by the size and velocity of the cutter integrated into the extruder (Featherstone, 2015). This texturization of the protein converts plant-protein into fibrous and expanded meat substitute, resembling meat-based products (Lyu et al., 2022). According to Riaz, (2011), TVPs are characterized by its distinctive spongy meat-like structure, resembling the chewy texture of meat when it is hydrated.

In order to achieve the desired texturization, raw materials with high protein concentration are needed in the TVP production; solvent-extracted flour or protein concentrate are used in the process. Moreover, other raw materials can also be added to obtain the desired properties, including nutritional balance and colour of the final products.

In the context of meat alternatives, TVP are used to imitate minced meat-based food items. For meat alternatives preparation, TVP crumbles are hydrated in a mixture of water and flavour-enhancing ingredients, following with mixing with binders (Riaz, 2011). The binders used in this process are commonly sourced from soluble proteins found in food products such as eggs, milk, or soy. However, other binding agents like starch and carboxymethylcellulose (CMC) may also be utilized. Afterward, the mixture is subjected to a pressing machine to be moulded into the desired shape, typically resembling patties or sausages.

There are several determining properties of TVP to be used as meat extenders including water/oil absorption, and textural properties (Riaz, 2011). Some research reported different extrusion parameter's effects on TVP properties. The effect of dry extrusion on protein isolate properties was studied by Beck et al., (2017). It was found that the expansion ratio of TVP decreases as protein content increases followed by the increase of the hardness. This is because of the changes in protein structure due to amount of protein denaturation and cross-linking. Moreover, in a review conducted by Day & Swanson, (2013), it was found that heat, shear, and moisture are independent variables in extrusion process that are influential for the changes of secondary, tertiary, and quaternary structure of protein. This is because, those variables affect the unfolding and crosslinking between protein due to thermal process involves in extrusion. In the research done by Osen et al., (2014), non-expanded TVP was obtained from pea protein containing more than 80% of protein without any addition of starch. Apart from the reduction in expansion ratio, protein denaturation during extrusion at high temperature is beneficial for protein digestibility. Lyu et al., (2022) explored the effects of screw speed and temperature in dry extruded TVP. It was reported that a reduction in screw speed with an increase in die temperature resulted in low SME and shear stress, two factors contributing to the expansion of TVP. Therefore, the increase of screw speed and temperature at extruder die will increase in expansion ratio. Regarding the microstructure of TVP, the increase in crosslinking which leads to protein aggregation was studied to be in parallel with an increase in temperature (Lyu et al., 2022).

2.3 Plant-protein fractionation

2.3.1 Air classification

Air classification is the most common dry fractionation technique for the production of plant protein concentrates. This process yields a fine protein fraction and a bigger size of starch-enriched fraction; in this technique, the air current being fed to the classifier chamber induces gravitational and centrifugal forces that separate the particles. The separation is based on their size and density of the fractions (Assatory et al., 2019; Martinez et al., 2016). This technique offers many advantages, including considerably low energy and water use, no chemical involved, and the preservation of native protein structure and functionality formation of disulfide bonds within protein (Luzzatto & Arese, 2018; Pelgrom et al., 2015; Vogelsang-O'Dwyer et al., 2020).

According to Martinez et al., (2016), protein concentrates produced by air classification have protein content of 50-65%, depends on the of raw material and processing condition. In a different study, the protein content of faba bean protein concentrate obtained through air classification fell within the

range of ~50-70% with the yield of around 70% (Vogelsang-O'Dwyer et al., 2020). The resulting protein content on the protein-enriched flour by air classification are considerably lower as compared to the wet fractionation. Moreover, air classification technique does not allow the removal of undesirable component such as oligosaccharides and antinutrients (Bhatty & Christison, 1984; Schutyser et al., 2015).

2.3.2 Wet fractionation

In wet fractionation, isolation of plant protein is done through aqueous extraction under alkali, acidic, or neutral conditions. This extraction is then followed by separation technique of the fractions such as isoelectric precipitation, ultra-filtration, neutralization or centrifugation (Lusas & Rhee, 1995). Wet fractionation combined with drying process offers higher protein content of up to 90%, as compared to the dry fractionation. However, the involvement of chemicals as well as the harsh processing condition (temperature and pH) leads to denaturation of protein, changing its chemical structure, thus losing its functionality. Moreover, with respect to the environment, wet fractionation generates chemical effluents and consumes substantial amount of water and energy especially related to the purification process (Assatory et al., 2019; Mondor et al., 2012).

Singhal et al., (2016) explored the effect of faba bean's genotype on the properties of protein isolate through wet fractionation under alkali condition followed by isoelectric precipitation and freeze drying. It was reported that the effect of genotypes was minimal and the level of protein in the protein isolate obtained was higher than 90% with the solubility of up to 98.83%. As for protein digestibility, it was found that protein isolate from wet fractionation has higher overall protein digestibility as compared to protein concentrate from dry fractionation process (Vogelsang-O'Dwyer et al., 2020). Liu, (2014) used modified wet fractionation to fractionate oat protein into 4 products enriched in protein, beta-glucan, starch, and other carbohydrates by using water as solvent followed by precipitation with an alcohol. The total recovery for protein and starch were 85.35-85.76% and 96.56%–100.18%, respectively.

2.4 Extrusion

2.4.1 Process

In food industry, extrusion process is generally referred as extrusion cooking. It is a process that uses an extruder, a machine equipped with a pierced piston with screws in a barrel to convey, mix, and cook the materials within the barrel. The operational characteristics of an extruder, including the level of shear, temperature, and its impact on the final product properties, vary

depending on the specific raw materials used and the desired attributes of the end product. This variation can range from low to medium to high shear conditions (Joy et al., 2012) During the extrusion process, the material undergoes mechanical and thermal treatment within the barrel before being compelled through a die with a specific shape. According to Vandenbossche et al., (2019), during extrusion process, there is a modification of internal structure of the matrix which dictates the final properties of the produced extrudates. This modification is dependent on the combination of shear stress and high temperature, as well as pressure.

Extrusion process in food industry is classified into two categories based on the moisture content: wet and dry extrusion. Typically, the moisture content of the feed wet extrusion is between 60-80%, while it is 10-30% in dry extrusion process (Vandenbossche et al., 2019). Wet extrusion cooking is broadly used in the production of meat alternatives. It involves texturization of vegetable protein, resulting in a product possessing a fibrous texture resembling animal meat. The product of wet extrusion is intended for direct consumption and is a complete (whole) meat replacement (Ryu, 2020). On the other hand, dry extrusion cooking is used to produce a wide range of ready-to eat cereals for breakfast or snack such as expanded cereals with airy, crisp, or crunchy texture and snacks with different shapes (Vandenbossche et al., 2019). Moreover, as regards to protein alternatives, dry extrusion is also broadly used for the production of meat extenders or ground meat alternatives (TVP) which need to be rehydrated prior to consumption.

According to Joy et al., (2012), extrusion process involves 5 systems:

a. Pre-conditioning
   Pre-conditioning is a step where raw material is pre-treated with water or steam. The purpose of this process is to achieve consistent hydration of particles, minimize retention times in the extruder and enhance the throughput. This, in turn, prolongs the lifespan of the equipment by reducing wear on barrel and screw components, while also decreasing the energy costs associated with the process (Vandenbossche et al., 2019).

b. Feeding system
   Feeding system typically consists of a container to load the raw material into the extruder barrel. To sustain an efficient and consistent operation of the extrusion process, it is crucial to establish a consistent and uninterrupted feeding system for the raw materials as extrusion process conditions is sensitive to feed rate changes, especially in the continuous production plan (El-Dash et al., 1983).

c. The screw/compression system
   Screw elements in extruder vary in shapes and quantity, with each segment being specifically designed for a particular purpose. Certain elements are responsible for conveying material into the extruder barrel, while others serve to facilitate kneading, induce backflow, and create shear forces. In extrusion process, screw configuration determines the cooking degree, starch gelatinization, and protein denaturation, as well as the final properties of the product (Joy et al., 2012). Therefore, suitable extruder screw is crucial in obtaining the desired characteristics of the products

d. The barrel/high pressure zone
   The barrel of an extruder is divided into three zones: feeding, kneading and high pressure. The sleeves encasing the screw is solid, and frequently equipped with jackets to facilitate the circulation of steam or superheated oil used for heating sources, or water or air for materials used for cooling such as water. This allows for precise temperature control in different zones of the extruder. Additionally, most sleeves are equipped with sensors for temperature, pressure (El-Dash, 1981).

e. The die and cutting mechanism
   The material under high pressure from previous zone is then expelled through the extruder die. Finally, due to the contact with ambient temperature, the extrudate expands to its final expansion property. The products expelled from die is then cut with a cutter. The exoansion size of the product is determined by the speed of the cutting blades. Cutting system has a purpose to enable the production of final products with consistent sizes (Joy et al., 2012).

2.4.2 Twin screw extruders

Single screw extruders are commonly used for a simple, and low-cost extrusion process. However, the growing need for a wider variant of superior quality of food products, which involve a broader range of cooking techniques, ingredients, and precise control over processing variables, has resulted in an increased use of twin screw extruders. Important factors possessed by twin screw extruder that is not present in single screw extruder are the level of intermeshing between the two screws and the direction of their rotation (Gray & Chinnaswamy, 1995).

A twin-screw extruder consists of two parallel shafts rotating inside a barrel. These shafts are fitted with interlocking screws that are arranged based on the chosen configuration. There are two types of rotation: co-rotating, where the shafts rotate in the same direction, or counter-rotating, where the shafts

rotate in opposite directions. This rotation direction is specific on the type of the machine (Vandenbossche et al., 2019).

In general, twin-screw extruders have three different of screw elements: (a) The direct pitch that is used for coverying; (b) The mixing elements (kneading disks), for distributing and dispersing the materials for a better mixing process; and (c) The reverse screws used mainly in the cooking section. These distinct screw elements effectively extend the residence time of the material within the extruder (Martin, 2016; Vandenbossche et al., 2019).

In extrusion, screw speed (rpm), feed rates, and temperatures throughout the barrel are the most common controlled parameters as it has relative impacts on the changes of biopolymer structural properties during the extrusion process. These parameters affect the properties of the extrudates due to the specific mechanical energy (SME) and specific thermal energy (STE) transferred to the material (Vandenbossche et al., 2019).

2.4.3 Effects on starch

The main effect of extrusion on starch is starch gelatinization. Gelatinization of starch is the disruption of the crystalline structure of starch, forming amorphous structures. Starch gelatinization starts with swelling followed by a rupture of starch granules, resulting in a partial hydrolysis of amylose and amylopectin to maltodextrin (Steel et al., 2012). This process is caused by the combination of shear stress and high temperature, as well as pressure applied to starch. In the experiment done by Wang et al., (2022), the starch content of cassava leaf reduced from 46% to around 40% after extrusion. This was because the expansion pressure broke down the external state of the grain as well as its molecular structure, cutting insoluble long chain of starch into short chains of sugar. Water present in the material as well as added water in this process act as plasticizer for starchy materials (flours), reducing its viscosity and mechanical energy which results in higher product density. The organized molecular structure of starch granules and water in extrusion process is destroyed by thermomechanical energy, resulting in molecular hydrolysis leading to the formation of continuous fluid melt (Vandenbossche et al., 2019).

Arêas, (1992) reported that the primary interaction between starch macromolecules during extrusion is attributed to electrostatic attraction among sugar residues. However, the application of shear stress, high temperature, as well as high pressure in the extruder leads to a simultaneous reduction of the interaction. As a result, the system undergoes a noticeable transition from a compressed powder state to a molten phase.

Furthermore, in the extrusion of starchy materials, the concentration of the material, particularly the feed's moisture content, give impacts on the effects of friction and flow behavior (Arêas, 1992). Another product parameter influenced by feed moisture related to the properties of extrudates is expansion ratio. Expansion ratio is a ratio of extrudate cross-sectional diameter as compared to extruder diameter of the die opening (Lyu et al., 2022a). During extrusion process, water within the material undergoes rapid evaporation due to the high temperatures and pressures. As the water transitions from a liquid to a gas state, steam bubbles are generated within the matrix of the material. These steam bubbles then expand, leading to the puffing and expansion of the extrudate (Guy, 2001). Relatively weak electrostatic attraction stabilizing starch molecule will result in high expansion ratio with a fragile structure (Arêas, 1992).

Moreover, cereal/legumes physical properties affect the final extruded products. Soft flour, such as corn flour, requires less mechanical energy in processing as it will create less mechanical energy between particles. Contrary, in specific types of cereals likehard wheat, durum wheat, and some varieties of barley, the presence of hard particles flour formed from starch and protein layers resulted in a higher energy required, thus generating more heat within the extruder (Steel et al., 2012).

2.4.4 Effects on protein

During dry extrusion, disulfide bonds of protein are cleaved, leading to polymerisation and reorganisation; the cross-linking process forming texturized protein occurs as the material flows inside the barrel (Arêas, 1992; Steel et al., 2012; Vandenbossche et al., 2019). Texturization of protein can be defined as a formation of three-dimensional structure of protein which occurs in three sequential steps: denaturation; reorganisation or polymerisation; and the subsequent solidification of the organized protein structure or gelation (González-Pérez & Arellano, 2009). Gelation in the extrusion is a result from heat treatment exceeding denaturation temperature of protein (Chen et al., 2011). **Figure 1** shows the texturization process of globular protein.

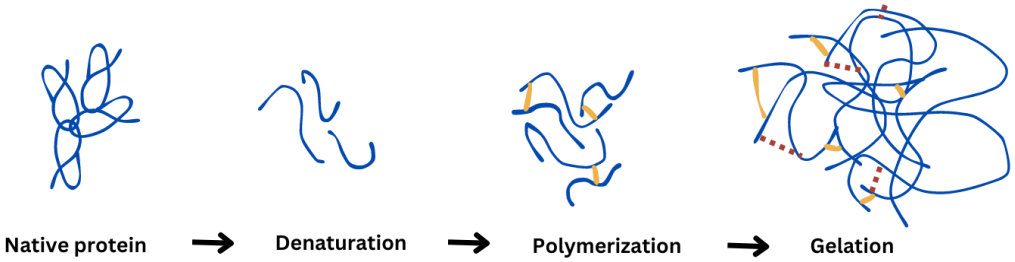

Figure 1. Schematic process globular protein texturization. Solid short yellow lines depict chemical bonding (disulfide bonds), while dotted red lines refer to physical interactions (hydrogen bonding, hydrophobic interactions, and electrostatic interactions) (Adapted from Sullivan et al., 2009)

During the gelation process, the stabilisation of the three-dimensional network that forms after extrusion cooking of protein is influenced by hydrophobic, cation-mediated electrostatic interactions, as well as covalent bonds (Arêas, 1992). Amino acid composition of the protein is the determining factor of the amounts of specific bonds formed. The varying amino acid composition between different materials and their complex interactions make the the study of extrusion parameters effect on protein often demonstrate a complex behaviour. However, it is evident that denaturation of protein during extrusion results in a decrease in solubility which favours digestibility and inactivation of anti-nutritional factors (Joy et al., 2012).

2.4.5 Novel extrusion technology for fractionation of plant protein

A novel dry extrusion process to fractionate plant-based raw materials to-protein-rich and starch-rich fraction has been recently patented (Nikinmaa et al., 2022). This process employs dry extrusion process to fractionate plant-based materials, producing a mixture of protein-rich and starch-rich extrudates. The two fractions can be separated, for example, by color, size, or hardness. Protein-rich extrudates produced by this process have protein content greater than 50%, which is considerably high for a fractionation process.

According to the patent (Nikinmaa et al., 2022), factors influencing good separation of two fractions are fat content in the raw materials of at least 5%, moisture content of at least 8%, depending on the source of raw material, and temperature of no more than 170°C.

The separation between protein-rich and starch-rich fraction in this process is hypothesized to happen as a result of phase separation occurring in high shear extrusion in the presence of low moisture. The separation between the two fractions is considered to result from the aggregation of protein due to

attractions between disulfide bridges, forming individual agglomerates (Nikinmaa et al., 2022).

2.5 Protein-polysaccharides interactions

Protein-polysaccharides interactions during extrusion of food biopolymers complex is necessary to be understood and as it substantially impacts the stability of biopolymer mixtures. Tolstoguzov (1997) categorized nonspecific protein-polysaccharides interactions into two distinct groups: attraction and repulsion between unalike macromolecules. These interactions have notable influence on the formation of complexes and the immiscibility of food polymers, thereby impacting the stability of both biopolymers. It was reported that protein-polysaccharides interactions; protein-protein interactions; and their interactions with solvent regulate the solubility and co-solubility of the biopolymers complex mixture, their pasting properties, and the complex behaviour of bioploymers at interfaces.

Mixture of proteins and polysaccharides exists either in a single stable phase or phase separated states (Tolstoguzov, 1997). Gibbs energy of mixing is the parameter influencing the occurrence of either phase. Positive value of Gibbs energy results in phase separated phase (thermodynamic incompatibility). System having positive Gibbs energy mostly consist of immiscible biopolymers. These biopolymers are differed in conformation, chain rigidity. and affinity towards solvent in the system (Tolstoguzov, 1997).Moreover, incompatible biopolymers also related to the volume occupation of the biopolymers as well as the repulsion between them. Thus, phase separation is sensitive towards the entropy factors from excluded volume of the macromolecules, This factors is influenced by the conformation and molecular weight of the macromolecules. Moreover, phase separation of a polymer complex occurs in high concentrated biopolymer mixture with a concentration higher than 20%. (Tolstoguzov, 1997; Van De Velde et al., 2015).

In the extrusion cooking process, protein is denatured due to high temperature and pressure as well as shear stress. In a study conducted by Tolstoguzov, (1993), it was reported that the incompatibility of protein-polysaccharides mixture increases as the protein molecules are denatured and aggregated which leads to increase in hydrophobic accessible surface and excluded volume of denatured protein.

2.5.1 Phase separation in protein-polysaccharide mixture

One of the protein-polysaccharide interactions in a biopolymer complex is phase separation. Phase separation in a protein-polysaccharide mixture is classified into two categories: segregative and aggregative phase separation.

Segregative separation occurs due to a repulsive interaction between two biopolymers. Repulsive interaction between two non-charged polymers containing 100 or more monomers overrules the very small mixing entropy of the mixture, leading to the instability of the mixture. This repulsive interaction is facilitated by the solvent. When two polymers with different hydrophilicities are present, there will be competition for hydration, with the more hydrophilic polymer binding more water. As a result, one phase contains the more hydrophilic polymer while the other phase contains the less hydrophilic polymer (van de Velde et al., 2015).

Hsu et al., (1974) used a computational modelling in identifying phase equilibria of two biopolymers system with a solvent based on Flory-Huggins theory and found that two polymers were completely miscible without solvent and concluded solvent addition to the system increased the entropy contribution and reduce Gibbs energy, leading to a phase separation. Other factors contributing to segregative phase separation is charge of the biopolymers. Bergfeldt et al., (1996) observed the effect of polymer charges on phase separation of a liquid-liquid system. It was found that the addition of electrolyte increased the entropy of mixing, leading to a more distinct phase separation. Segregative separation can also occur in the mixture of polymers having the same charge (van de Velde et al., 2015). In this case, the difference in charge density is the primary factors leading to the phase separation. In this mixture, phase instability is only observed at a high concentration in which absence of hydration induces some particular interactions.

In the case of two oppositely charged biopolymers, association of the biopolymers results in aggregative phase separation (**Figure 2**). Many factors contributing to aggregative phase separation, including the presence of electrolytes. The association of two biopolymers in aggregative phase separation is not only a result of attraction of two polymers possessing opposite charges, but also the effect of the release of counterions from the formation of neutral entity of two oppositely charged group of polymers, which increase the entropy of mixing, as found by Kayitmazer et al., (2013) in a modelling and simulations of protein-electrolytes system. In aggregative phase separation, two phases formed are polymers-rich phase and polymer-depleted phase.

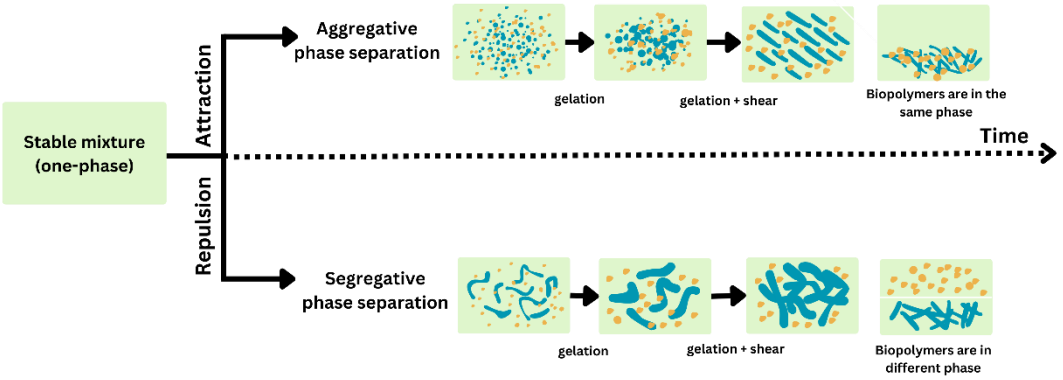

Figure 2. Schematic illustration of phase separation in protein-polysaccharide mixture (Adapted from Stieger & Van de Velde, 2013; Van De Velde et al., 2015)

2.5.2 Response to shear

During the extrusion process of food biopolymer, shear influential factor contributing to the conformational changes of the biopolymers. In extrusion-aided plant protein fractionation, shear generated from screw was expected to have a substantial role in the biopolymers separation. Walkenström et al., (1999) oberseved the effects of shear stress on phase separation of a mixture of biopolymers. It was found that shear treatment reduced the viscosity of aggregated suspension and led to a reduction of the aggregate size. Moreover, in research conducted by Wolf et al., (2000), it was observed that anisotropic structure in biopolymer mixture is a result of controlled shear flow during the gelation process of the dispersed phase. These anisotropic particles can align in such a way that leads to shear thinning with the presence of shear stress. Subsequently, as the shear stress continues to increase, the material become insensitive to additional increase of shear rate. This insensitivity then leads to the instability of the system, resulting in phase separation. These two layers differ in viscosity and the separation occurs because of the concentrated strain rate in the least viscous layer (Van De Velde et al., 2015).

Shear banding is a phenomenon that occurs in a melted biopolymer during extrusion process with the simplest example of shear thinning under increasing strain rates (Van De Velde et al., 2015). Schubert et al., (2004) studied a shear-induced phase separation in micelles system. It was found that shear-induced system can lead to phase separation with an increase of shear stress induces shear thinning due to increasing strain rates. It was observed that micellar samples became turbid after the shear treatment and underwent

phase separation. The study stated that phenomenon occurred for solutions with long-ranged concentration fluctuations which is correlated with shear banding.

2.5.3 Two-phase system in the extrusion process

Extrusion-aided plant fractionation involves in a system of complex biopolymers and utilizing the incompatibility of starch-protein complex. Arêas, (1992) suggested an extrusion model for macromolecules considering the complex biopolymer melt as a two-phase system. This model could be applied to all types of polysaccharides and proteins mixture which might also suitable for two-phase separation in extrusion-aided faba bean fractionation optimized in this thesis. It was proposed that insoluble molecules are located at the center of the system as the material flows in the extruder barrel (**Figure 3**). In the protein-rich system, these molecules can exist as insoluble entities or protein aggregates formed during processing, and the barrel temperature is responsible for the proportion of these insoluble material.

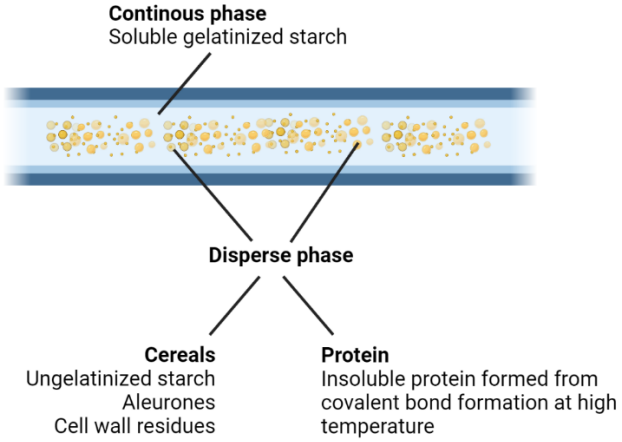

Figure 3. Illustration of biopolymer melt phase inside the extruder barrel (Adapted from Arêas, 1992)

The degree of solubility of protein before and after extrusion process can be classified into 5 states, which is determined semi-quantitatively from the extrudates (Arêas, 1992):

State 1	Protein soluble in buffer alone
State 2	Protein insoluble due non-covalent interactions
State 3	Protein insoluble due to disulfide interactions
State 4	Protein insoluble due to a combination of disulfide and non-covalent interactions
State 5	Inderterminate, all protein unaccounted for is classified in this state, calculated by difference

3 Research material and methods

3.1 Materials

3.1.1 Faba bean flour

Crushed faba bean was purchased from Suomen Viljava Oy, Finland. The samples were milled once using an Alpine Fine Impact Mill 100 UPZ-1b with a 0.5 mm sieve and rotor speed of 10000 rpm.

Faba bean used for pre-trial works was Faba bean whole flour purchased from Suomen Viljava Oy, Finland.

3.1.2 Rapeseed oil

Rapeseed oil used was produced by Avena Kantvik Oy, Finland

3.2 Methods

3.2.1 Extrusion-aided protein fractionation

Low moisture extrusion for separation of protein-rich and starch-rich fraction was performed using APV MPF 19/25 co-rotating twin screw extruder from Baker Perkins Group Ltd, Peterborough, UK with a K-tron twin screw feeder K-MV-KT20. The screw used was 19mm in diameter and having L/D ratio of 25:1. The screw profile was composed of a 290 mm twin lead feed screw, a 20 mm half feed screw, 20 mm kneading element, 20 mm half feed screw, 60 mm kneading element, 25 mm half feed screw, 25 mm kneading element, and 30 mm discharge element to the exit (**Figure 4**).

Extruder barrel was composed of four temperature zones (Zone 1-5): a feeding zone (Zone 1) and four temperature-controlled zones (Zone 2-5) heated by an electric heater and cooling by water circulation. The temperature profiles were set to 80 (Zone 2), 95 (Zone 3), 125-143 (Zone 4), and 135-153°C (Zone 5).

Faba bean flour and rapeseed oil were blended with a cutter Metos vcb-62 400v3. The mixing process was done in a 1.5kg batch of flour with slowly pouring the oil. The cutter speed was set to the highest (II), and once all the oil was added, the mixing was continued for 2 minutes. Water addition level, screw speed of, and Zone 4 and Zone 5 barrel temperature were manipulated during extrusion process, according to the experimental design. The process

temperature **Table 2** was barrel temperature of Zone 5 while the temperature of Zone 4 was kept 10°C lower than Zone 5.

During the extrusion process, a 2-bladed cutter of the extruder was used at speed setting of 2.2 to reduce the size of the product into small granules. The product consisted of a mixture of dark-coloured protein enriched granules and light-coloured starch-enriched granules. The extrudates were then collected on trays and immediately dried in an oven drier with air circulation at 95°C for 10 minutes.

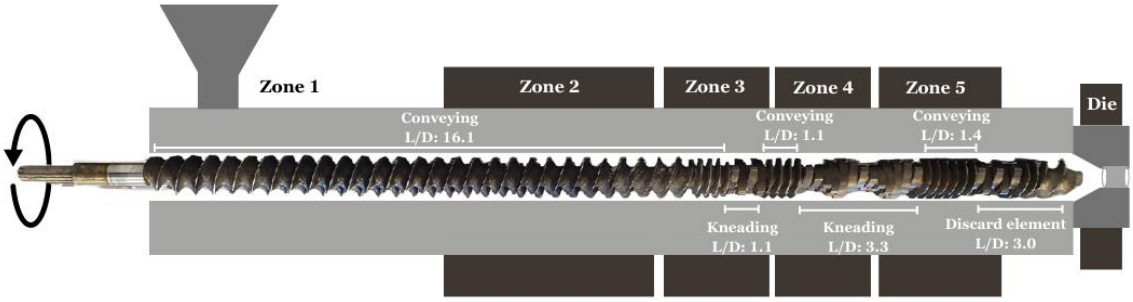

Figure 4. Screw configuration of the twin-screw extruder profile used in the experiments

3.2.2 Separation of fractions after the extrusion

Following the extrusion process, a combination of sieving and manual separation was employed to separate the protein-rich and starch-rich fractions from one another. First, the finest particles (mainly consisting of protein enriched particles) were separated by sieving. Retsch Vibratory Sieve Shaker As 200 Control was used with a mesh size of of 3.15 mm in size (Santalo - Sohlberg AB). The product was sieved in batches of 120 g and the shaker was set for 2 minutes with interval of 60 seconds.

Following the sieving process that separated the finer particles, the larger granules containing both starch-enriched and protein-enriched crumbles underwent manual separation. The separation of the larger crumbles was based on the colour difference of the fractions: protein-enriched crumbles were dark in colour and the starch-enriched crumbles were light in colour. Finally, the “protein-rich fraction” was produced by combining the fine fraction and the dark-coloured large crumbles. The “starch enriched fraction” was composed of the large light-colored crumbles.

3.2.3 Grinding of samples for analysis

Grinding samples for techno-functional analysis was performed by using ZM 200 Ultra Centrifugal Mill, Retsch® with sieve size of 0.5 mm. Approximately 30g of sample was subjected to the grinder with the speed of 10000 rpm.

3.2.4 Total protein content

The total protein content of the ground and liquids samples was calculated based on the nitrogen content (N) according to the Dumas combustion method (AACC Approved Methods of Analysis, 11th Ed. Method 46-30.01. Crude Protein - Combustion Method. Approved November 8, 1995. Cereals & Grains Association, St. Paul, MN, U.S.A. http://dx.doi.org/10.1094/AACCIntMethod-46-30.01) using a Protein/Nitrogen Analyser (rapid MAX N exceed, Elementar Analysensysteme GmbH, Langenselbold, Germany) with a conversion factor of 6.25. Each sample was analysed in triplicates (200 mg × 3 and 3 mL × 3, for ground and liquid sample, respectively).

3.2.5 Protein yield

Protein yield was determined by comparing the total protein content of the protein-rich fraction (consisting of fine fraction and dark color crumbles) to the total protein content of the raw material. The calculation was done based on the following equation

$$Protein\ yield\ (\%) = \frac{(PD \times WD) + (PF \times WF)}{PR \times WR} \times 100\ \% \quad (1)$$

PD : protein content of the dark crumbles (% dm)
WD : weight of the dark crumbles (g dm)
PF : protein content of the fine fraction (% dm)
WF : weight of the fine fraction (g dm)
PR : protein content of the raw material (% dm)
WR : weight of the raw material (g dm)

3.2.7 Total protein solubility

Total protein solubility of ground samples was performed according to the method of Rekola et al., (2023) with some modifications. Protein extraction of protein-rich fraction (1.5 g) and faba bean flour (0.75 g) was conducted in buffe solutions (30 ml, 30 s, 13,500 rpm, room temperature) using Silent-Crusher M, Heidolph Instruments, Schwabach, Germany. Following the extraction, the samples were centrifuged (30 mins, 10000×g, 20°C Heraeus Multifuge X1R centrifuge, Thermo Fisher Scientific, Osterode am Harz, Germany) and the supernatants were subjected to total protein content analysis as previously described. Analysis was carried out in triplicates.

Protein solubility (%) was calculated based on the following equation:

$$Protein\ solubility\ (\%) = \frac{PN \times VB}{PS \times WS}\ x\ 100\% \qquad (2)$$

PN : protein content of supernatant (%)
VB : volume of extraction buffer (mL)
PS : protein content of the sample (% dm)
WS : weight of the sample (g dm)

3.2.8 Bulk Density

Bulk density measurement was performed according to the method of Hong et al., (2022) with minor modifications. Bulk density was analysed only for the fractions separated manually (the fine fraction separated by sieving was not included in the measurement). Protein-rich and starch-rich dry crumbles were filled in a 250 mL measuring cylinder and was shook gently twice to eliminate the interspace between crumbles. The weight of the sample per 250 mL was recorded. The bulk density was expressed as weight per volume (g/L). Each measurement was done in triplicate.

3.2.9 Stereo-microscopy

ZEISS SteREO Discovery.V8 with Axiocam 705 color camera was used to observe protein-rich fraction and starch-rich fraction. 1 × magnification with grey filter was used for all samples.

3.2.10 Water Absoption Capacity and Oil Absorption Capacity

Water /oil absorption capacity (WAC/OAC) analysis were performed following the method of Hong et al., (2022) with some modifications. WAC was performed by dispersing 1 g of ground sample in 10 mL deionized (DI) water

in a pre-weighed 15 mL centrifuge tube. The mixture was then vortexed thoroughly for 30 s and allowed to stand for 5 min at RT. Centrifugation of the mixture was done at 3000×g for 30 min using Heraeus Multifuge X1R centrifuge, Thermo Fisher Scientific, Osterode am Harz, Germany. After centrifugation, the supernatant was discarded and the tube containing precipitate was inverted for 5 minutes to drain the residual water before re-weighing (A2). As for OAC, 1g (O0) of sample was mixed with 10mL rapeseed oil in a pre-weighed 15 mL centrifuge tube (O1). The mixture was then allowed to stand for 30 min at RT before centrifugation at 3000×g for 30 min. After centrifugation, the supernatant was discarded and the tube containing residue was inverted for 10 min and the remaining oil residue was removed. The final weight of the sample was then measured (O2).

The WAC and OAC were presented as weight of water and oil absorbed (g) per sample weight (g), using following equations. Each analysis was performed in triplicate.

$$WAC\ (g\ water/g\ sample) = \frac{W2 - W1 - W0}{W0} \quad (3)$$

$$OAC\ (g\ oil/g\ sample) = \frac{O2 - O1 - O0}{O0} \quad (4)$$

W0/O0 : Weight of the sample (g dm)
W1/O1 : pre-weighted centrifuge tube (g)
W2/O2 : final weight of the samples (g)

3.2.11 Rehydration capacity

Rehydration properties of protein-rich fraction were performed according to Hong et al., (2022) with some modifications. The analysis was done in room temperature water and boiling water for 2 h. The final weight of the sample at 1 h; 1.5 h; and 2 h were recorded. Three grams of sample was rehydrated in 45 mL DI water (1:15 solid to liquid ratio) at RT for rehydration capacity measurement at RT. As for rehydration capacity of sample in boiling water, the sample was soaked in boiling water and put in an oven at 105°C. The timer was set once the temperature of the sample reached 90°C. Following rehydration, the sample was drained to remove the excess water for 5 minutes at RT. Analysis was performed in triplicate.

Rehydration capacity was calculated based on the following equation:

$$RHC\ (g\ water/g\ sample) = \frac{WAR - WBR}{WS} \quad (5)$$

WAR : weight after rehydration (g)
WBR : weight before rehydration (g)
WS : weight of the sample (g dm)

3.2.12 Textural properties of rehydrated extrudates

Textural properties of protein-rich extrudates were characterized (TA.XT plus, Stable Micro Systems Ltd., United Kingdom). Sample that has been rehydrated for 1.5 h as described above was placed in a stainless-steel cylinder with 25 mm in diameter and 30 mm in height. The cylinder was filled with sample until it reached the same height as the cylinder. TPA was conducted by one compression test using a cylinder prober with 20 mm in diameter at a strain compression of 30% with 10 g trigger force, 5 mm distance (target of deformation), 1 mm/s pre-test speed, 2 mm/s post-test speed, and 2 mm/s test speed. The textural attribute of hardness (the peak force during the compression) was recorded. Analysis was done in triplicate.

3.2.13 Viscosity

The viscosity of the samples was analyzed using Rapid Visco Analyser (RVA) Super 4 (Newport Scientific Pty Ltd., Warrierwood, Australia). The analysis was carried out by placing ground sample (10%dm) in a canister and mixed with DI water, forming 28g of slurry. The initial temperature at the beginning was set to 25°C. The slurry was then mixed by stirring for 10 seconds for thorough dispersion at 160 rpm. The same speed was set for the remaining RVA test. The temperature of 25°C was hold for 5 minutes before ramping up to 95°C with the increase of 10°C/min. After holding time for 5 minutes at 95°C, the slurry was cooled down to the initial 25°C with the same speed and was held for another 5 minutes. The viscosity after 5 minutes holding at 25°C, after 5 minutes holding at 95°C, and the final viscosity were recorded. Each measurement was performed in triplicate.

3.2.14 Statistical analyses

Data were analyzed using one-way ANOVA using IBM SPSS Statistics Data Editor. Duncan's multiple range test was used for mean comparisons, and $p < 0.05$ was considered as the threshold level of significance. Least significant difference (LSD) values were calculated at 5% level of significance. Pearson correlation coefficients were determined to investigate the association between variables.

4 Results and discussion

4.1 Study outline

The workflow of the study is presented in **Figure 5**. Dry extrusion process was performed for the production of protein-rich and starch-rich. Pre-trial works were performed in order to decide the concentration of oil used for the optimization and method of controlling feed rate during extrusion. Following this, extrusion for optimizations in 17 conditions based on the experimental design were conducted to evaluate which combination of conditions favouring protein and starch separation. The analysis of protein content in protein-rich fraction and starch-rich fraction as well as the calculation of protein yield were done subsequent to the extrusion. Based on the protein content of the protein-rich fraction and protein yield, three optimized conditions were chosen for producing the protein-rich and starch-rich fraction that then will be evaluated for their techno-functional properties.

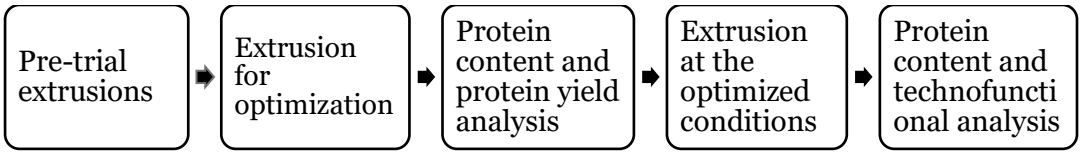

Figure 5. Workflow of the study

4.2 Pre-trials

4.2.1 Feed rate control

Pre-experimental investigations were performed to explore effective methods for controlling the feed rate. It was found that the feed rate exhibited varying degrees of instability when different types of flour were used. Therefore, feed rate control was done by weighting the extrudates exiting the die for 2 minutes before the sample collection and subsequently adjusting the feed rate based on the observed measurements. When the sample was in the range of accepted weight (around 55-62g/min), a 10-minute sample collection was initiated. Subsequently, the collected samples were weighed, with the acceptable range for sample collection being 560-620g per batch which was equivalent to 58±3 g faba flour/min. Moreover, the addition of flour to the feeder bowl at intervals of 2 minutes was done in order to control the stability of the feed rate. The schematic illustration of extrusion aided faba bean is shown in **Figure 6**.

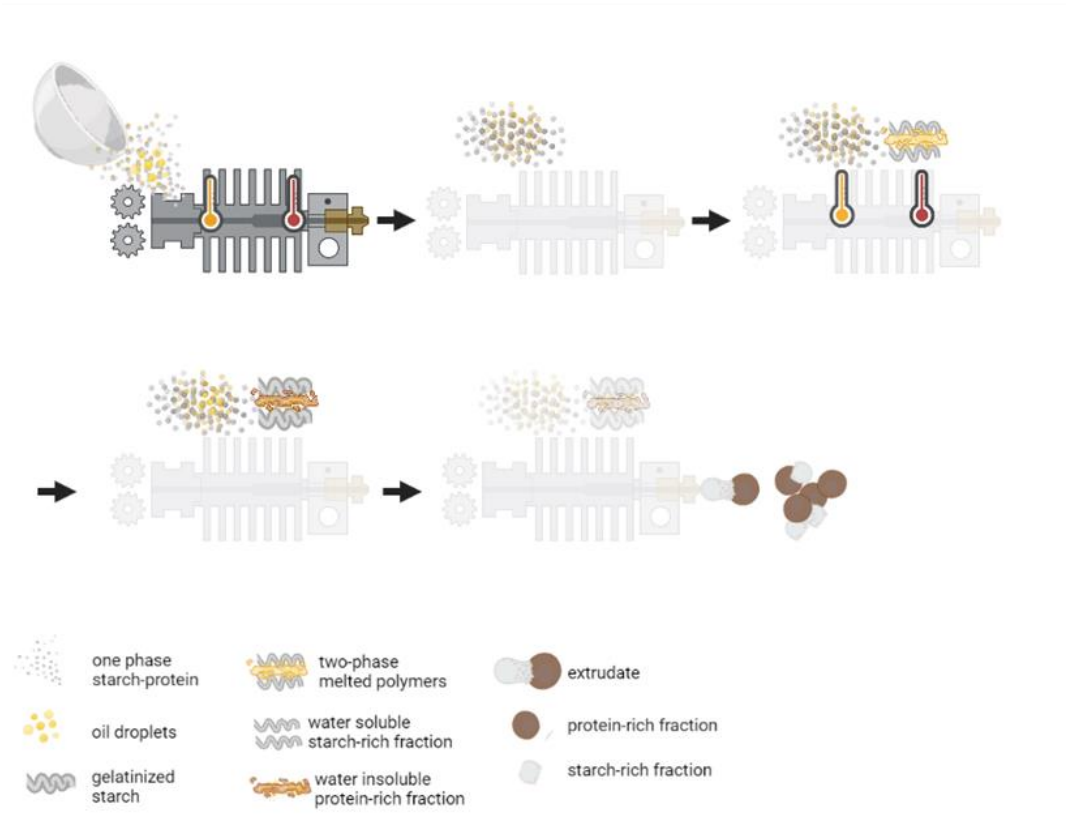

Figure 6. Schematic presentation process of the extrusion-aided faba bean protein fractionation process in the experiment

4.2.2 The effect of fat content on starch-protein separation

Some pre-trial works were performed to determine the concentration of oil (4,6,8%) added to the flour for a starch-protein separation to happen. The evaluation was done by observing the protein-rich fraction and starch-rich fraction in terms of color and size. The concentration of oil favouring distinct differences (size and color) of the two different extrudates were chosen.

In this experiment, increasing the fat content to 6% led to a more noticeable contrast in color and size between the starch-rich and protein-rich fractions (**Figure 7**). Additionally, the protein-rich fraction exhibited a higher protein content of 50.62% and protein yield of 84.75% compared to the lower fat content (4%), which resulted in a protein content of 45.45% and a protein yield of 52.30%. The results of this work align with the findings reported by Arêas, (1992) regarding the solubility of protein in defatted lung flour with varying residual fat content (0.5% and 6.5%). It was found that the presence of higher fat residue in the flour plays a crucial role in determining the specific interactions between proteins in the extrudates (Arêas, 1992). Sample with higher residual fat showed an increase of the amount of insoluble protein due to

disulfide and non-covalent interactions and an increase in the aggregated protein.

Moreover, corresponding to the higher protein yield obtained from the sample having higher fat content in this work, Tolstoguzov, (1993) found that the aggregation of protein leads to phase separation between protein. This phenomenon occurs due to an increase in the hydrophobic exposed surface area and the excluded volume of the denatured protein. This finding was also supported by Nikinmaa et al., (2022) that the higher concentration of oil might promote hydrophilic-hydrophobic separation between protein and starch.

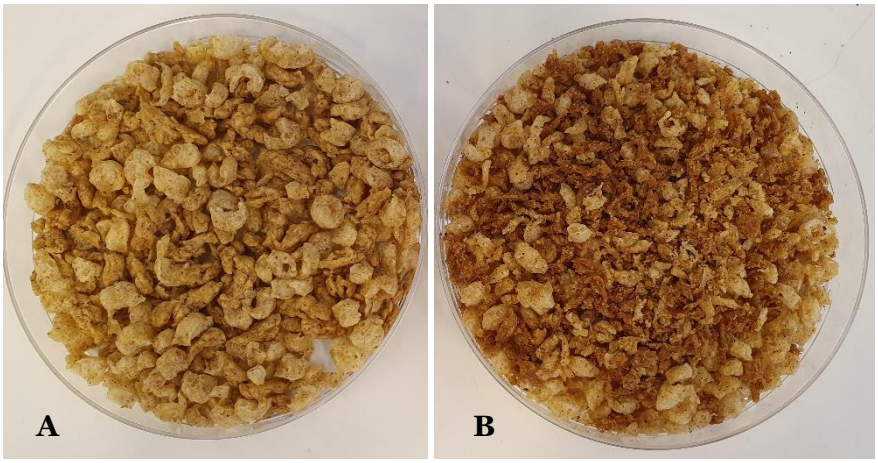

Figure 7. Mixed fractions of extruded faba bean flour with fat content of 4% (A); and mixed fractions of extruded faba bean flour with fat content of 6% (B)

Moreover, according to the research conducted by Van de Velde et al. (2015), the repulsive interaction between protein and starch during segregative phase separation is facilitated by the solvent. Polymer having higher hydrophilicity (starch) binds more water and the less hydrophilic polymer (protein) will be concentrated and mediated by oil/lipid. That findings indicated that phase separation between two biopolymers depends on the affinity between the different biopolymers and the solvent in the system. The net repulsion between the biopolymers arises when there is a positive Flory-Huggins interaction parameter for the interactions between biopolymer1 and biopolymer2 (χ_{23}). Additionally, the interactions between the solvent and biopolymer1 (or biopolymer2) have a detrimental effect on both the biopolymer1-biopolymer2 interactions and the solvent-solvent interactions. (Zeman & Patterson, 1972). Therefore, the addition of higher fat is potentially associated with better separation of protein-rich and starch-rich fraction in the extrusion-aided faba bean fractionation, as it is a good solvent for insoluble protein, while water is a good solvent for starch.

Despite the fact that a higher fat concentration favored protein-starch separation, sample with 8% fat the addition was not feasible to extrude as leaking of oil was observed during extrusion process. Therefore, based on the better results in separation of protein-rich fraction and starch-rich fraction as compared to the lower fat content, fat addition of 6% was chosen in this thesis study.

4.3 Optimization of extrusion conditions

Upon establishing the method for controlling the feed rate during extrusion and determining the optimal fat content for effective starch-protein separation, the next step involved optimizing the extrusion conditions. The experiment was designed using Central Composite Face design (CCF) under Response Surface Methodology (RSM). MODDE®13 from Sartorius, Germany was used for modelling of the experimental design as well as RSM results. The design was a two-level model with three center points. The factors (independent variables) selected were moisture addition during extrusion (2-4 ml/min), screw speed (300-450 rpm), and temperature (135-153°). Dependent variables were protein content and protein yield of the protein-rich fraction. The experimental runs were randomized to minimize bias from systematic observation of the responses. The experimental design resulted in 17 combinations of parameters.

The results on protein content of protein-rich fraction and protein yield as well as the corresponding protein content of starch-rich fraction from extrusion at 17 conditions are summarized in **Table 2**. ANOVA and regression coefficients are summarized in **Table 3**. In all models, the value of predicted variation (Q^2) was greater than 0.5 and the difference between Q^2 and R^2 was less than 0.3, indicating that the model was a good model. Moreover, none of the models showed lack of fit.

The response contour of regression model on protein content and protein yield of protein-rich fraction as well as protein content of starch-rich fraction are shown in **Figure 8**. Factors that had an impact on the protein content of the protein-rich fraction were process temperature and screw speed. These factors had negative correlation with protein content in which the lower the screw speed and temperature, the higher protein content of protein-rich fraction was obtained. Contrary to the other independent variables, added moisture did not have a substantial effect on the protein content of the protein-rich fraction.

On the other hand, the response contour of protein yield shows that added moisture had the most ubstantial influence on the protein yield as

compared to the other variables (screw speed and temperature). The results showed that as the added moisture decreases, the protein yield increases.

Table 2. Effects of extrusion conditions/factors (moisture, screw speed and temperature) on the product responses (protein content of protein-rich fraction and protein yield and the corresponding protein content of starch-rich fraction). The data is presented as mean values with standard deviation indicated by ±.

No	Run order	Factors (actual and coded values*)			Product responses		
		Moisture (mL/min)	Screw speed (rpm)	Temperature (°C)	Protein-rich fraction		Starch-rich fraction
					Protein content (%)	Protein yield (%)	Protein content (%)
1	13	2 (-1)	350 (-1)	135 (-1)	68.77 ± 0.56	78.20 ± 1.14	10.50 ± 0.18
2	11	4 (+1)	350 (-1)	135 (-1)	71.13 ± 1.16	46.80 ± 0.90	22.18 ± 0.75
3	15	2 (-1)	450 (+1)	135 (-1)	67.69 ± 0.72	83.09 ± 0.12	11.28 ± 0.51
4	12	4 (+1)	450 (+1)	135 (-1)	66.35 ± 0.74	57.77 ± 0.33	18.33 ± 0.32
5	4	2 (-1)	350 (-1)	153 (+1)	64.44 ± 0.73	82.54 ± 0.40	6.32 ± 0.19
6	9	4 (+1)	350 (-1)	153 (+1)	68.33 ± 0.39	65.35 ± 0.44	15.85 ± 0.96
7	3	2 (-1)	450 (+1)	153 (+1)	58.97 ± 0.71	75.28 ± 0.09	9.76 ± 0.32
8	2	4 (+1)	450 (+1)	153 (+1)	58.66 ± 0.51	66.62 ± 0.54	13.58 ± 0.50
9	5	2 (-1)	400 (0)	144 (0)	68.18 ± 0.80	77.17 ± 0.42	9.49 ± 0.50
10	7	4 (+1)	400 (0)	144 (0)	66.68 ± 0.78	62.73 ± 0.09	17.90 ± 1.13
11	6	3 (0)	350 (-1)	144 (0)	70.34 ± 0.57	76.98 ± 0.25	13.34 ± 0.31
12	8	3 (0)	450 (+1)	144 (0)	63.25 ± 0.65	72.11 ± 0.17	14.04 ± 0.64
13	14	3 (0)	400 (0)	135 (-1)	70.31 ± 0.62	70.27 ± 0.19	12.33 ± 0.08
14	1	3 (0)	400 (0)	153 (+1)	62.95 ± 0.88	71.85 ± 0.23	11.53 ± 0.48
15	10	3 (0)	400 (0)	144 (0)	66.21 ± 0.57	71.35 ± 0.64	12.64 ± 0.22
16	16	3 (0)	400 (0)	144 (0)	71.52 ± 0.99	87.35 ± 0.36	10.08 ± 0.77
17	17	3 (0)	400 (0)	144 (0)	74.14 ± 0.51	82.21 ± 0.25	12.80 ± 0

* coded values are in parenthesis

Table 3. Analysis of variance for the fit of experimental data of extrusion-aided faba bean fractionation to response surface model

Regression	Sum of squares		
	Protein-rich fraction		Starch-rich fraction
	Protein content	Protein yield	Protein content
R^2	0.849	0.884	0.936
R^2 adjusted	0.656	0.735	0.853
Q^2 (predicted variation)	0.588	0.539	0.541
P	0.032	0.014	0.002
RSD (%)	1.649	3.445	4.888
Lack of fit	0.934no	0.814no	0.729no

no., no lack of fit

It is worth to mention that there was an inverse correlation between the protein content and protein yield of the protein-rich fraction (**Figure 8**). This is evident from the opposite effect of the responses to the independent variables. Specifically, while moisture content had a great influence on protein yield, it did not have a substantial impact on protein content. Conversely, screw speed and temperature that showed a slight positive correlation with protein yield, had a considerable negative correlation with protein content. This difference in responses suggests that the independent variables play a complex role in determining both the quantity of protein recovered from the raw material (protein yield) and the concentration of protein within the yield (protein content).

The inverse relationship between protein content and protein yield in this extrusion-aided fractionation process might be the results from a competition between segregative phase separation and protein gelation. According to Doublier et al., (2000), in the protein-polysaccharide complexes, phase separation occurs simultaneously with the gelation of one or both components. This phenomenon was observed when a mixture of globular protein and polysaccharides underwent thermal treatment, leading to the gelation of the globular protein. In the context of extrusion-aided protein fractionation of faba bean, it is noteworthy that globulin, a type of globular protein, is the highest protein component of the raw material. Consequently, during this thermal process, the competition between phase separation and protein gelation might occur. The prominence of either phase separation or protein gelation was observed to be dependent on the extrusion condition. These distinct processes associated with product responses in this study was hypothesis to be characterized by protein yield for phase separation and protein content for protein gelation. This hypothesis can be supported by the physical appearance of the protein-rich and starch-rich fraction in **Figure 9** in which starch-rich fraction produced in the conditions favouring protein content

demonstrate the presence protein rich-fraction within it. On the other hand, the starch-rich fraction produced from the conditions favouring protein yield did not contain protein-rich fraction. However, additional studies are required to establish a definitive relationship between gelation and phase separation phenomenon with protein content and protein yield analyzed from this extrusion-aided fractionation method.

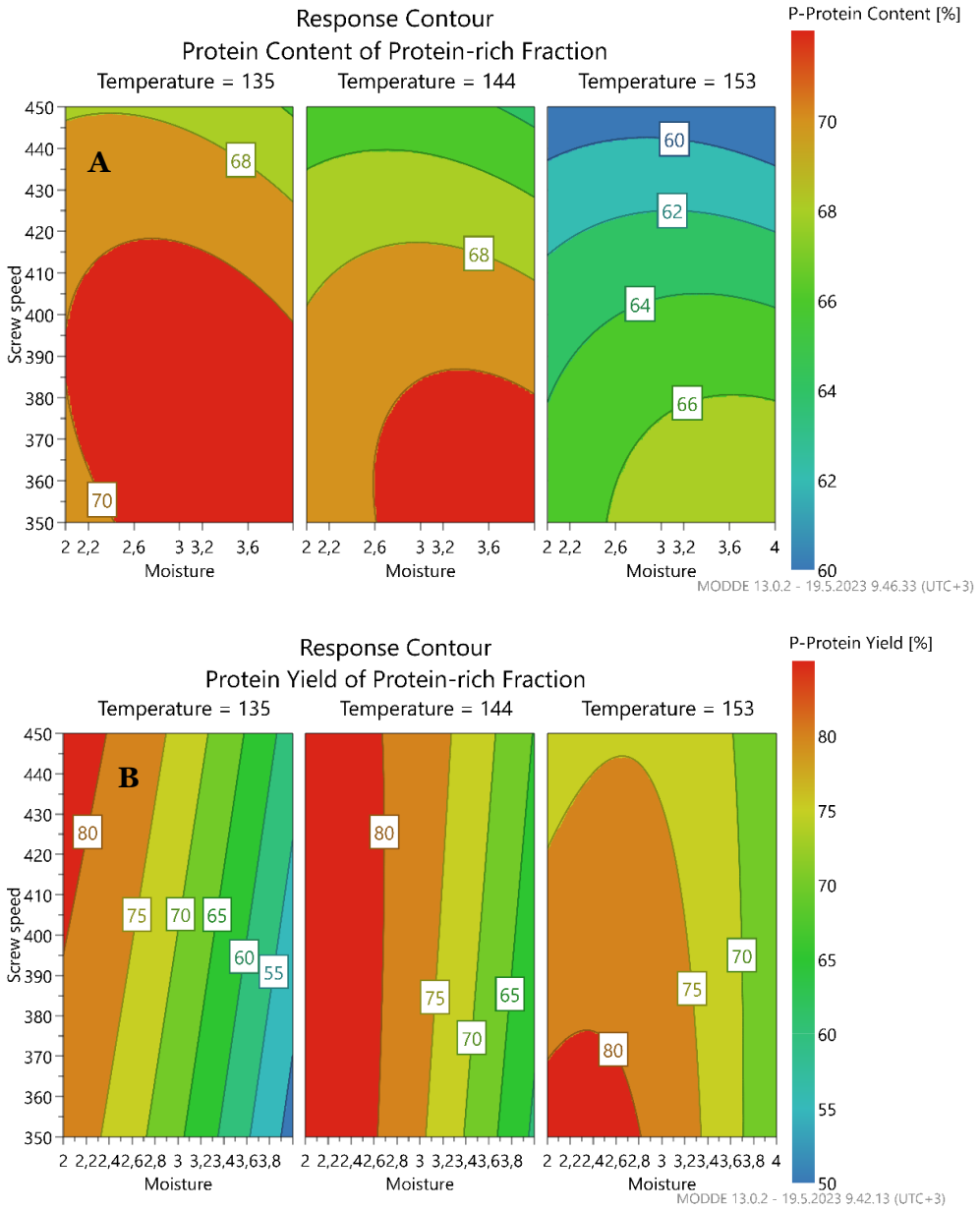

Figure 8. Response contour based on the regression model of dry extrusion-aided faba bean fractionation showing the impact screw speed, moisture, and temperature, on the protein content of the protein-rich fraction (A) and protein yield (B)

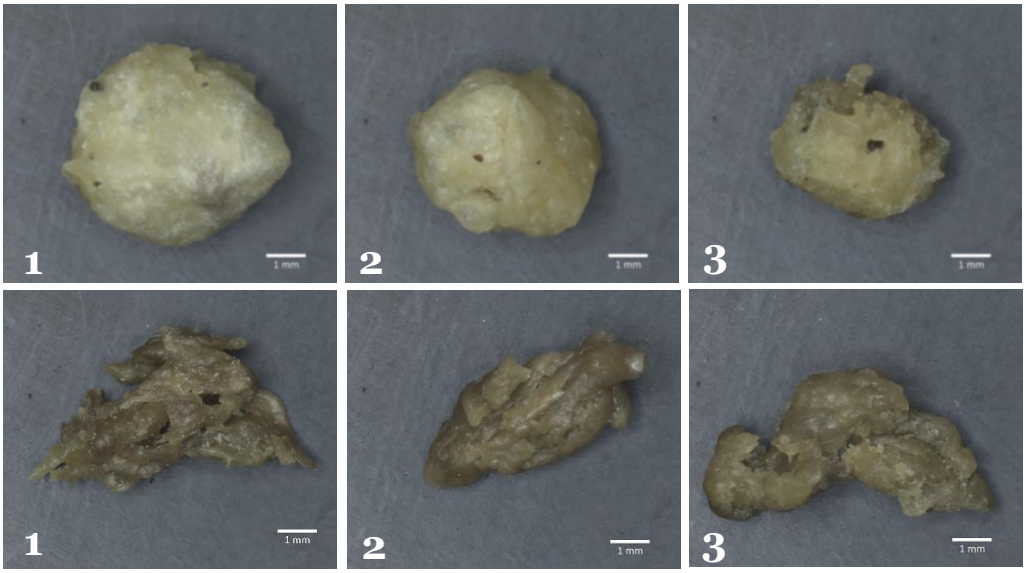

Figure 9 Starch-rich fraction (upper) and protein-rich fraction (lower) under stereo microscope with 1× magnification. The order of the numbering is based on the samples numbering on optimized conditions: highest protein yield (1), 'set point' (2), and highest protein content (3)

Subsequently, based on the response contours, three extrusion conditions with the objectives of highest protein content, highest protein yield, and the 'set point' was generated using MODDE® optimizer feature. Set point is a condition chosen from the best compromise of the respective factors, calculated based on the consideration of both limits of the responses and the acceptance limit.

All extrusion results obtained from the three optimized conditions correspond to the predicted values generated by MODDE®, indicating a successful optimization and a good repeatability of the process (**Table 4**)

The protein content of protein-rich fractions produced from the optimized extrusion process was in the range of 71-77% with the protein yield of 60-85%. As compared to the dry fractionation process, the extrusion-aided fractionation process resulted in a higher protein content, while as compared to the wet fractionation process, it had higher protein yield. Dry fractionation of faba bean reported by Martinez et al., (2016) resulted in a flour having protein content of up to 66% with protein recovery reaching 91%. However, the air classification was conducted two times. As for wet fractionation, Jeganathan et al., (2023) conducted a study about mild wet fractionation

process of faba bean flour using water with the addition of salt as solvents. The study reported the protein content of up to 92% with the protein yield of 24%.

Moreover, the extrusion-aided fractionation using faba bean was not only better as compared to the dry fractionation and wet fractionation. But the protein content of the protein fraction was higher than protein content of TVP in the market (51-73%) (Hong et al., 2022).

4.3.1 The effect of extrusion temperature

The response contours show that extrusion temperature in the area of around 140°C resulted in both higher protein content and protein yield (red area) (**Figure 8**). This finding aligned with the previous observations; Lyu et al., (2022) found that higher barrel temperature led to an increase in protein crosslinking and disulfide bonds formation due to protein denaturation. However, according to K. S. Liu & Hsieh, (2008), excess heat during in extrusion process could result in a partial disruption of non-covalent bonds (hydrophobic force, electrostatic force, hydrogen bond) and covalent bonds (S-S bond, peptide bond). The texturization of protein during extrusion process can be divided into three subsequential steps: denaturation, rearrangement or polymerization and gelation (González-Pérez & Arellano, 2009). Therefore, it is possible that extrusion-aided separation process at low temperature did not facilitate protein gelation, resulting in a low protein content and protein yield. Conversely, the crosslinking between protein was disrupted by excess heat. Thus, the optimal temperature for the extrusion process lies within a moderate range.

As regards to the extrusion at the optimized conditions, it was suggested that there is a negative correlation between barrel temperature and protein content (**Table 4**). According to Beck et al., (2017), the increase of barrel temperature results in shorter residence time of the sample inside the extruder barrel and lower SME. Therefore, it could be possible that high extrusion temperature resulted in lower protein content of the protein-rich fraction because there was not enough time for the protein to form aggregates (the last step of protein texturization).

Conversely to the protein content, barrel temperature resulted in the positive effect on protein yield (**Table 4**). This is because extrusion process at higher temperature could increase the degree of denaturation in the protein, resulting in an increase of surface area of the protein as proteins unfold. According to K. & Bandyopadhyay, (2012), unfolded conformations of protein expose more of their reactive sites, resulting in more chances of interactions with other components in the material. This may offer an explanation of the

higher yield and lower protein content, as the weight of protein-rich fraction was higher due to the presence of other component binding to the protein.

4.3.2 The effect of moisture

The quantity of moisture added during the extrusion process showed a negative correlation with protein yield, whereas it did not substantially affect protein content (**Figure 8**). According to Van De Velde et al., 2015, segregative phase separation, or thermodynamic incompatibility, is observed at high polymer concentrations when there is insufficient hydration of water. Our study's findings suggested a potential alignment with this finding, demonstrating that the sample with lower moisture addition (2 mL/min) resulted in a higher protein yield compared to the sample with 4 mL/min water addition.

On the other hand, higher moisture addition during extrusion in this study resulted in higher protein content of the protein-rich fraction, corresponding to the results of Beck et al., (2017). An increase in moisture during low-moisture extrusion of material rich in protein leads to a rise in the formation of hydrogen-bonded aggregates, or other non-covalent crosslinking aggregates.

4.3.3 The effect of screw speed

The screw speed of the extruder showed a negative correlation with the protein content of the protein-rich fraction, while it did not considerably impact protein yield (**Figure 8**). This can be attributed to the fact that the screw speed determines the residence time of the samples within the extruder barrel, with higher screw speeds resulting in shorter residence times (Beck et al., 2017). Similar to the effect of temperature, a shorter residence time led to incomplete protein aggregation, resulting in a lower concentration of protein in the protein-rich fraction.

As per the findings reported by Lyu et al. (2022) and Yeh & Jaw., (1999), an elevation in screw speed during extrusion operations leads to an increase in SME. This increase in SME will then lead to a notable conversion of mechanical energy into self-heating energy. The increase of SME due to a higher screw speed might explain the rationale behind a higher screw speed in the 'set-point' condition as compared to the condition favouring highest protein content and the condition favouring highest protein yield. The higher screw speed could potentially facilitate the generation of additional energy, which helps prevent blockage. Simultaneously, it might also help to add more heat for protein aggregation to occur.

Table 4. Predicted optimized conditions and actual results of extrusion-aided faba bean protein fractionation

Sample	Condition	Protein-rich fraction				Starch-rich fraction		
		Protein content (%dm)			Protein yield (%)	Protein content (%dm)		
		Prediction	Actual result		Prediction	Actual result	Prediction	Actual result	
1	Temperature : 147 °C Moisture : 2 mL/min Screw speed : 350 rpm	68.05	71.02	± 0.14[a]	83.76	84.85 ± 0.16[c]	8.28	7.85	± 0.02[a]
2	Temperature : 140 °C Moisture : 2 mL/min Screw speed : 384 rpm	70.67	73.47	± 0.19[b]	81.25	79.97 ± 0.20[b]	10.11	12.66	± 0.61[b]
3	Temperature : 140 °C Moisture : 4 mL/min Screw speed : 350 rpm	72.45	76.78	± 0.29[c]	57.71	59.84 ± 0.23[a]	19.95	20.58	± 1.3[c]

Sample 1,2, and 3 is optimized condition with maximized protein yield, a set point (optimal balance between high protein yield and high protein content), and maximized protein content, respectively.

4.4 Techno-functional properties analysis

4.4.1 Bulk density

Bulk density measurement in the characterization of TVP provides analyzes the overall expansion of the extrudates and the conformational changes of the protein (Brishti et al., 2021). Moreover, bulk density measures the amount of air cells in extrudates (Lyu et al., 2022). An increase in expansion due to high amount of air cells in the extrudates, decreases the bulk density. Our study's findings are consistent with the previous findings (Beck et al., 2017; Hong et al., 2022; Lyu et al., 2022). As evidenced in **Table 5**, the starch-rich fraction of Sample 3, having the least expansion based on the observation, exhibited the highest bulk density, indicating a denser structure of extrudates (**Figure 9**). Moreover, bulk density of protein extrudates also have a correlation with their protein content. Higher protein content as a result of high degree of protein cross-linking of extrudates has been found to be corelated with stronger structure of extrudates, which prevents expansion and increases bulk density (Philipp et al., 2017). This study's findings agree well with the findings of Hong et al., (2022) which was also supported by significant positive correlations between protein content of the fraction and their bulk density ($r = 0.964$, $p<0.01$; and $r = 0.913$, $p<0.01$, for protein-rich and starch-rich fraction, respectively), wherein both starch-rich and protein-rich fraction of Sample 3, having the highest protein content, result in the highest bulk density.

The effects of extrusion parameters on expansion of extrudates related to the bulk density has been broadly researched. According to Lazou & Krokida, (2010), higher barrel temperature reduces material's viscosity, leading to the formation of air cells inside the material which then results in the expansion of the extrudates. In this work, starch-rich fraction of Sample 1, being processed at the highest temperature (147°C) exhibited the highest expansion (**Figure 9**). In addition, as regards to the screw speed, Sample 2 (384 rpm), processed with higher screw speed and at the same temperature, was more expanded than Sample 3 (350 rpm) (**Figure 9**). This result agrees well with Liu et al., (2005) which reported that there is a positive correlation between screw speed and expansion of the extrudates due to an increase in mechanical energy and shear, resulting in an elevated molecular flow.

Hong et al., (2022) compared physicochemical and functional properties of commercial TVP from different plant sources. The average bulk density of pea protein TVP was reported at 246 g/L, while it was 320 g/L for soy protein. The bulk density of both protein-rich and starch-rich fraction produced in this study were higher than the findings by Hong et al., (2022). This might be because of the difference in the degree of texturization of the protein. Moreover, Lyu et al., (2022) observed the impact of extrusion parameters on the physicochemical and textural characteristics of textured vegetable protein (TVP) composed of a mixture of soy protein isolate, wheat gluten, and corn starch. The study findings revealed a wide range of bulk densities for the TVP samples, varying between 70-560 g/L. The bulk density of protein-rich fraction and starch-rich fraction of faba bean in this study fell between the range observed by Lyu et al., (2022).

As regards to the effect of moisture on the bulk density, the results of extrusion-aided faba bean fractionation as well as the previous findings agreed that extrusion at lower temperature and high moisture content results in extrudates with high densities, while higher temperature and lower moisture results in extrudates having low densities. According to Yu et al., (2013), TVP extruded with high moisture content absorbs more water. This will result in less water being evaporated at the extrusion die, minimizing the air cells formed, and resulting in denser extrudates.

Table 5. Bulk density of protein-rich and starch-rich fraction

Sample	Bulk density (g/L)	
	Protein-rich fraction	Starch-rich fraction
1	453.31 ± 0.75[a]	417.71 ± 2.66[a]
2	498.52 ± 0.85[b]	508.57 ± 1.05[b]
3	522.96 ± 0.29[c]	542.33 ± 0.10[c]

4.4.2 Protein solubility

The effect of extrusion-aided protein fractionation on protein-protein interactions was evaluated by determining the solubility of protein-rich fractions. Three extraction buffers were used in this analysis: of 100mM phosphate buffer (P) (NaH2PO4, Na2HPO4; pH 7.5), a buffer solution PS (P + 1% SDS) and a buffer solution PSD (PS + 50mM DTT).

As compared to the raw material, all protein-rich samples had lower solubility in all extraction solutions. Moreover, higher protein solubility was observed in protein-rich fraction extracted with PSD (36.81-38.55%), followed by PS (15.60-22.74), and P (5.33-8.81%) (**Figure 10**).

The use of buffer alone was expected to dissolve denaturated protein that is not forming stable network (Arêas, 1992). In the protein solubility measurement of extrusion-aided faba bean fractionation, there is a significant different between the protein solubility of the three different samples in P in which the higher protein contained in the sample, the lower is the protein solubility (**Figure 10**). This is also supported by a significant negative correlation between protein content of the extrudate and its solubility in P ($r = -0.941$, $p<0.01$). This result is in accordance with Arêas, (1992). The heat and shear energy during extrusion process induced the protein gelation and aggregation due to the formation of disulfide bonds, covalent bonds, and hydrophobic bonds, linked to the extrudate three-dimensional structure. Therefore, the increase in protein concentration in the protein-rich fraction might indicate more protein being involved in a complex protein-protein interactions, and a lower proportion of loosely structured protein (non-aggregated protein).

As regards to the solubility in PS, SDS in the extraction solution weakens non-covalent bonds such as hydrogen, ionic, and hydrophobic interactions within protein aggregates (Iwaki et al., 2020). Protein extractability of Sample 3 in PS was significantly lower than Sample 1 and 2, indicating a higher formation of protein aggregation (**Figure 10**). This observation is consistent with the significantly higher protein content (76.78%) present in Sample 3, which is also emphasized with a significant negative correlation with protein content of the sample ($r = -0.854$, $p<0.01$). This result corresponds with Li & Lee, (2000) that the solubility of protein is dependent on its molecular weight, the decrease in protein solubility during extrusion in the extracting solvents indicate the increase of the molecular weight of the protein which might be the result of the protein association during extrusion. Furthermore, this high molecular weight protein is not easily disrupted by the extraction solvent (Lin et al., 2000). This explains why samples having higher protein content in this study had lower protein solubility.

As regards to the solubility of protein in PSD, DTT it is commonly used to break disulfide bonds within protein polymer (Rekola et al., 2023). Therefore, the combined action of DTT and SDS disrupted both non-covalent bonds and the disulfide bonds. Consequently, this led to no significant difference in solubility among the three samples, as all bonds were effectively cleaved. This result indicated that the difference in protein content might be

attributed to the variation in non-covalent bonds present within the proteins in the protein-rich fraction.

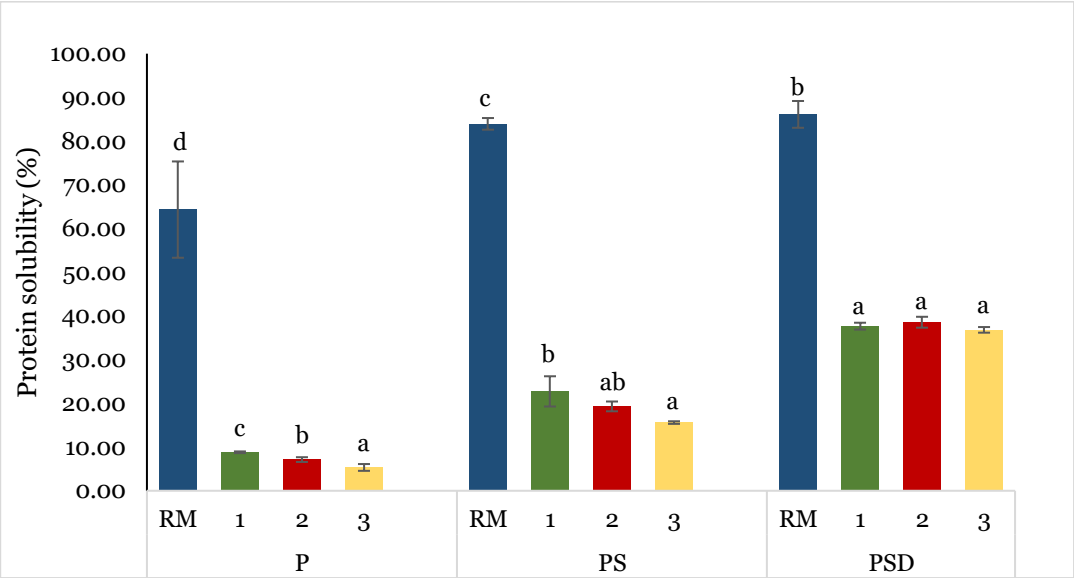

Figure 10. Effect of extrusion-aided fractionation on protein solubility of the raw material (RM); protein-rich fraction produced from the conditions favouring the highest protein yield (1), set point (2), and highest protein content (3) by extraction solvents; 100 mM phosphate buffer (P), 100 mM phosphate, 1% SDS buffer (PS), and 100 mM phosphate, 1% SDS and 50 mM DTT (PSD). Protein solubility was expressed as percentage of total protein. Different letters (a–d) in the same extraction solution indicate statistically significant differences ($p<0.05$) between samples based on LSD test. Samples and raw materials were analyzed separately.

4.4.3 Water Absorption / Oil Absorption Capacity (WAC/OAC)

WAC/OAC indicate the amount of water or oil can be absorbed by samples at the macromolecular level (Hong et al., 2022). These properties are indicators to assess the suitability of the products to be integrated into water-based or oil-based food products.

WAC and OAC values of protein-rich and starch-rich fraction is summarized in **Table 6**. WAC values of protein-rich fraction were in the range of 2.34-2.59 g/g dm and significantly different between Sample 1 and Sample 3. The WAC of commercial TVP made of soy, pea, and wheat and are in the range of 1.5-2.9 g/g with pea TVP having the average of 2.1 g/g (Hong et al., 2022). Therefore, the WAC of the protein-rich fraction produced from extrusion-aided fractionation of faba bean was in the range of WAC of commercial TVP in the market.

The OAC values of protein-rich fraction ranged between 0.94-1.01 g/g dm; the OAC of Sample 1 and 2 were significantly higher than Sample 3 (**Table 6**). Moreover, there is a significant negative correlation between OAC and protein content of the protein rich fraction ($r = –0.849$, $p<0.01$) and significant positive correlation ($r= 918$, $p<0.01$) with protein yield. This result corresponds with the hypothesis that protein in the sample with higher protein yield were more denaturated with higher surface area due to protein unfolding. The more exposed area of the denaturated protein might bind more with oil than the closed one.

As for starch-rich fraction, the WAC of Samples 2 and 3 were significantly different in which Sample 3, having higher protein content, exhibited lower WAC values (**Table 6**). However, WAC value of Sample 1 was not significantly different with Sample 2 and 3. The OAC value of three samples were not significantly different since protein within the starch-rich fraction might be the one binding with oil while in these fractions, the more dominant component was starch.

Table 6. WAC/OAC values of protein-rich and starch-rich fractions. Different lowercase letters indicate significant difference among samples means within the same column ($p < 0.05$)

Sample	Protein-rich fraction		Starch-rich fraction	
	WAC (g/g dm)	OAC (g/g dm)	WAC (g/g dm)	OAC (g/g dm)
1	2.34 ± 0.05^{a}	1.01 ± 0.06^{b}	3.50 ± 0.16^{ab}	0.88 ± 0.05^{a}
2	2.42 ± 0.09^{ab}	1.01 ± 0.02^{b}	3.72 ± 0.17^{b}	0.89 ± 0.04^{a}
3	2.49 ± 0.03^{b}	0.94 ± 0.02^{a}	3.32 ± 0.05^{a}	0.98 ± 0.07^{a}

4.4.4 Rehydration capacity

Rehydration capacity (RHC) is a characterization method of TVP, related to the amount of water retained by the intact extrudates upon rehydration. In the context of meat alternatives, RHC is an important factor which affects the meat-like texture of plant-based meat alternatives (D. Webb et al., 2020). RHC measurement in this study was performed in RT water at RT and in boiling water with temperature being kept at around 100°C. The objective of this measurement was to investigate the characteristics of extrudates upon rehydration at RT as well as under boiling conditions. The results showed (**Figure 11**) that boiled extrudates held more water than extrudates rehydrated at RT. This phenomenon might arise due to the thermal effects induced during boiling, which result in the expansion of the dense structure of the extrudates, enhancing their ability to hold water.

RHC of the protein-rich fraction in this study ranged from 1.57-1.82 g/g dm at RT and 2.68-3.08 g/g dm at boiling condition (**Figure 11**). It is shown that the fractions with higher protein content and more compact structure have higher RHC than the ones with lower protein content as it was supported by the Pearson correlation of the relationship between RHC with protein content both at boiling and room temperature (r = 0.892, $p<0.01$; r = 0.687, $p<0.05$, respectively). In this study, protein-rich fraction with higher protein content possessed a denser and compact structure. This result aligns with Hong et al., (2022) findings that more compact products were able to retain higher amount of water, while lower RHC occurred in more porous and fibrous structure. This is because the presence of a higher number of air cells within a porous protein network may facilitate the drainage of water, as increased porosity facilitates water release due to the gravitational force (Hong et al., 2022). It is also depicted in **Figure 9** that protein-rich fraction of Sample 1 has more void structure than Sample 2 and 3. Besides the number of pores of the extrudates, the void space also plays crucial role to drain water (Webb, 2021)

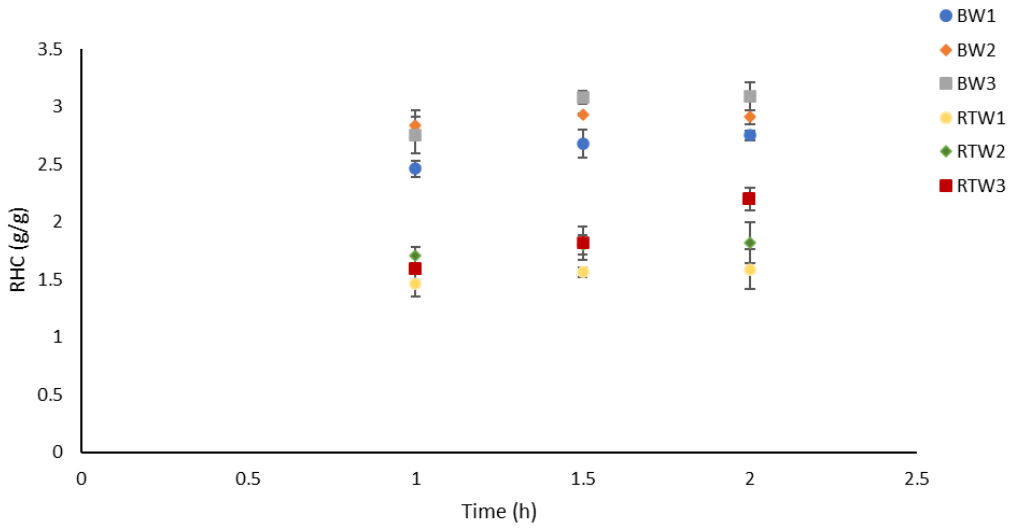

Figure 11. Kinetics of the rehydration capacity (RHC) of protein-rich fraction in boiling water (BW) and RT water (RTW). Number 1, 2 and 3 following BW and RTW correspond to the samples number of samples produced from optimized extrusion conditions: maximized protein yield (1), 'set point' (2), maximized protein content (3)

Table 7. Rehydration capacity (RHC) of protein-rich fraction in room temperature and boiling water at 1.5h of rehydration. Different lowercase letters indicate significant difference among samples means within the same column ($p < 0.05$)

Sample	RHC in RT water (g/g dm)		RHC in boiling water (g/g dm)	
1	1.57	± 0.12[a]	2.68	± 0.04[a]
2	1.80	± 0.01[b]	2.93	± 0.08[b]
3	1.82	± 0.16[b]	3.08	±0.15[b]

Hong et al., (2022) studied the physicochemical properties of commercial TVP made of soy, wheat and pea at RT. The RHC obtained were 2.8, 3.1, and 2.9 g/g, respectively. The RHC of protein-rich fraction in this study were considerably lower. This might be due to higher degree of texturization of the protein which also shown by higher bulk density as compared to findings of Hong et al., (2022).

4.4.5 Textural property

Texture is one of the most important factors in food product development. In the context of texturized plant-protein, the primary objective of developing plant-based alternatives is to achieve a desirable texture that closely resembles meat. The evaluation of textural property for the protein-rich fraction of faba bean was conducted using samples that had been rehydrated. As it is shown in **Figure 11** the rehydration rate increases with time, but it showed a plateau after 1.5h of rehydration. Therefore, samples rehydrated at 1.5h were chosen to be characterized for their textural properties. The textual properties in terms of hardness of the protein-rich fraction are summarized in **Table 8**. 'Hardness is the maximum force required to attain a defined deformation' (Breene & Barker, 1975). According to Samard & Ryu, (2019) hardness of TVP is an indicator of the level of protein texturization whereby a higher protein content in the raw materials leads to an increase in texturization caused by cross-linking of the protein. As the degree of texturization higher, the extrudates' expansion is inhibited which leads to a higher hardness.

There was no significant difference between samples both rehydrated at RT and at boiling temperature (**Table 8**). However, there was a significant negative correlation between RHC of protein-rich fraction at RT and its hardness ($r = -0.698$, $p<0.05$), agreeing with previous observations (Hong et al., 2022; Osen et al., 2015). It was evident that extensive hydration of extrudates results in a softer texture, decreasing the hardness. The hardness of commercial TVP analyzed by Hong et al.,(2022) was 917, 736, and 512 g for soy, pea, and wheat TVP, respectively. Therefore, the hardness of protein-rich fraction produced from extrusion-aided fractionation was in the range of commercial TVP.

Table 8. Textural property (hardness) of protein-rich fraction. The same lowercase letters indicate there is no significant difference among samples means within the same column (p > 0.05).

Sample	Hardness of samples rehydrated in RT water (g)	Hardness of samples rehydrated in boiling water (g)
1	845.33 ± 79.10[a]	500.67 ± 92.04[a]
2	753 ± 83.81[a]	369 ± 31.43[a]
3	763.67 ± 178.16[a]	444.33 ± 138.36[a]

4.4.6 RVA pasting properties

Pasting properties evaluate the the pasting capacity of of flour/starch. Assessing the pasting properties helps to understand the functional attributes of food products, including thickening and gelling capacity and texture-improving characteristics (Aidoo et al., 2022; Dey et al., 2021). In extrusion-aided faba bean fractionation, RVA pasting properties of starch-rich fractions and their corresponding protein-rich fraction were measured. The pasting properties measured were the viscosity in the end of first 25°C, after heating at 95°C for 5 minutes, and the final viscosity. As it is shown in **Figure 12** there was an instant thickening of all starch-rich fraction at 25°C (cold-water swelling capacity) while the raw material (native starch) shows a plateau until before the temperature was increased. This instant thickening of starch fraction at comparatively lower temperature is defines as cold-water swelling capacity. Cold-water swelling capacity is a distinctive property of pre-gelatinized starch. Y. Liu et al., (2017) evaluated the properties of pregelatinized starch produced by of improved extrusion cooking technology as compared to the native starch of rice starch. They found that the pre-gelatinized rice starch was easier in absorbing water and swelled faster than the native starch.

The pasting properties of the starch-rich fractions produced from three different extrusion conditions were significantly different at 25°C, 95°C, and final viscosity (**Table 10**). The starch with lower protein content had a higher viscosity than that of the one having higher protein content. Hong et al., (2022) found that the lower viscosity in extrudates is highly correlated to the protein contained in which higher insoluble protein content led to lower viscosity. This is because higher protein content associates with a more complete texturization which leads to a weaker binding to the water. Moreover, the final viscosities of the starch-rich fractions were found to be negatively correlated with protein content of the fractions ($r = -0.958$, $p<0.01$). In addition, the viscosity of the starch-rich fraction at 95 °C and at final measurement showed a positive relationship with WAC ($r= 0.781$, $p<0.05$; $r= 0.775$,

p<0.05, respectively), agreeing with findings of Hong et al., (2022) and Osen et al., (2014) in which the higher WAC of fractions indicates a higher ability to absorb water which results in higher viscosities

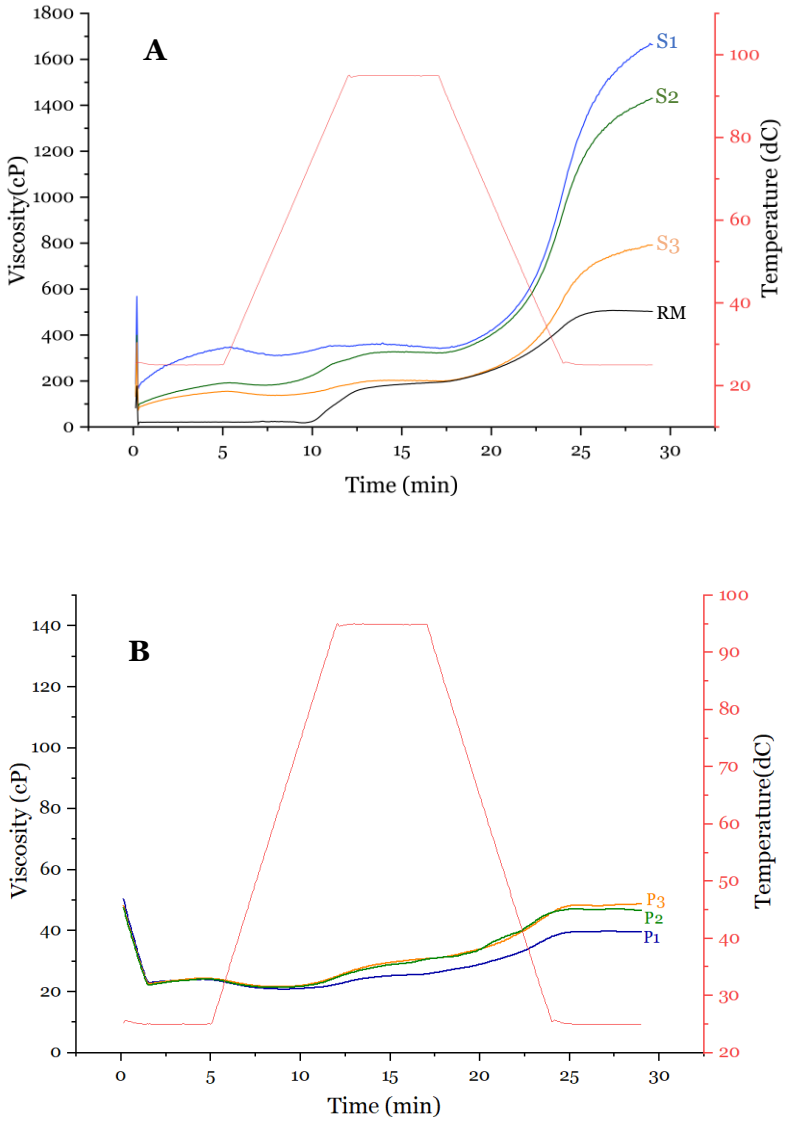

Figure 12. Pasting properties of (A) starch-rich fraction; (B) protein-rich fraction from extrusion aided Faba bean fractionation.

Table 9. Viscosity values of protein-rich fraction at the end of temperature cycle and final measurement

Sample		Viscosity at 25°C (cP)	Viscosity at 95°C (cP)	Final viscosity (cP)
Protein-rich fraction	1	24.33 ± 1.53[a]	25.67 ± 1.15[a]	39.33 ± 1.53[a]
	2	25 ± 1.73[a]	30 ± 1.00[b]	45.33 ± 1.15[b]
	3	24.67 ± 1.15[a]	30.67 ± 0.58[b]	48.67 ± 1.15[c]

Table 10. Viscosity values of starch-rich fraction at the end of temperature cycle and final measurement

Sample		Viscosity at 25°C (cP)	Viscosity at 95°C (cP)	Final viscosity (cP)
Starch-rich fraction	RM	21.67 ± 0.58[a]	194.33 ± 7.23[a]	502.67 ± 20.56[a]
	1	341.67 ± 13.43[d]	344.33 ± 15.31[c]	1664 ± 179.72[d]
	2	190.33 ± 5.03[c]	322.33 ± 8.08[b]	1430.33 ± 9.81[c]
	3	154.67 ± 6.43[b]	199.67 ± 6.66[a]	791.67 ± 23.71[b]

5 Conclusions

The state-of-the-art dry extrusion-aided plant protein fractionation recently patented by VTT has proven to be a successful method for fractionating faba beans into protein-rich and starch-rich fractions. The addition of oil played an important role in the succeed of this process. Moreover. the optimization process performed in this thesis highlighted the differentiation between extrusion conditions favouring high protein content and those leading to high protein yields of the protein-rich fraction. These differences could be due to competition between segregative phase separation and protein gelation. Notably, under optimal extrusion conditions, the protein-rich fraction obtained demonstrated a higher protein content compared to commercial TVP, while maintaining comparable techno-functional properties.

Furthermore, the findings showed the protein content of the fractions plays a critical role in influencing their techno-functional properties. This relationship could be closely linked to the extent of protein cross-linking and denaturation induced by the extrusion process. Nevertheless, further investigation is required to fully comprehend the scientific relationship between extrusion conditions, protein content, and the corresponding techno-functional properties.

Additionally, this study found that the extrusion-aided fractionation process produced starch-rich fraction which demonstrates a property of cold-water swelling capacity. This finding opens up possibilities for utilizing the starch-rich fraction from extrusion-aided fractionation process in various food applications.

Moreover, from a sustainability perspective, the elimination of protein-enriched flour as a raw material in this process present a more sustainable approach compared to conventional TVP production. Overall, this research offers valuable insights into the optimization of extrusion process conditions for plant protein fractionation and paves the way for the development of innovative and eco-friendly plant protein alternatives, promoting a more sustainable future in the food industry.

6 Future perspectives

There are some components that were not analysed in this study including fibres and fat that might have an impact on the biopolymers behaviour during extrusion and also the techno-functional properties of produced protein-rich and starch-rich fraction. Therefore, a comprehensive analysis regarding the composition of protein-rich fraction and starch-rich fraction is needed to provide a better understanding of the effects of the extrusion conditions to protein, polysaccharides, and their interactions, including phase separation of the two biopolymers.

This thesis highlighted the potential of protein-rich fraction to substitute TVP as it had comparable properties with commercial TVP based on the literature. However, applications study is needed to be performed. Additionally, investigating its potential utilization in alternative protein sources and fortifying existing food products with the protein-rich fraction could provide innovative solutions for increasing protein content in diets.

As regards to the starch-rich fractions, further exploration is needed to investigate its applications in the development of pre-gelatinized starch for use in various food products. Understanding its functionality and potential benefits in terms of texture, stability, and sensory attributes would be valuable as extrusion-aided plant protein fractionation could produce two fractions having superior properties.

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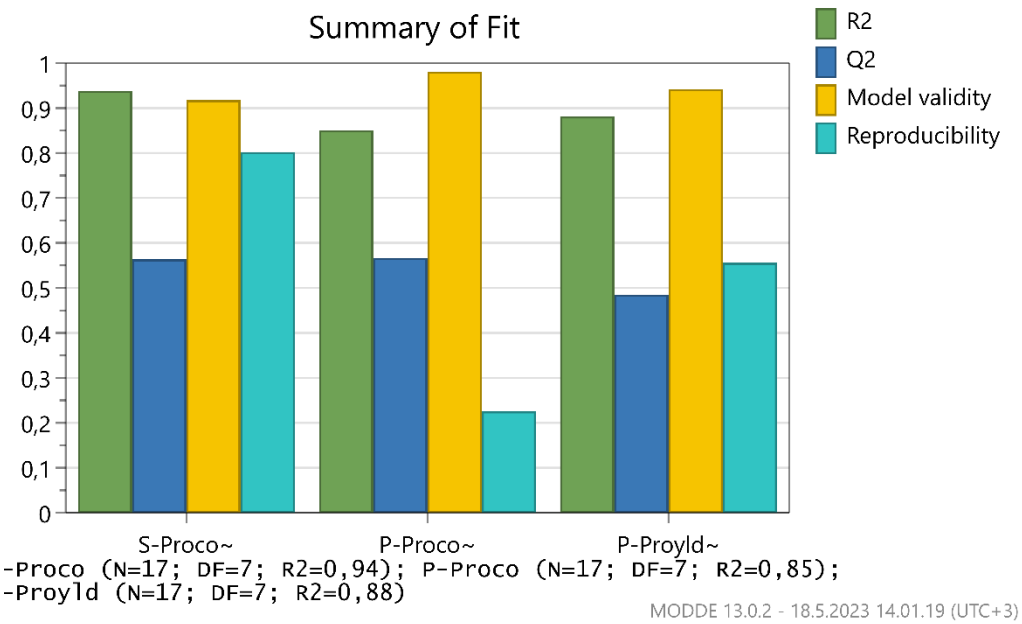
8 Annex



Annex 1. Summary of fit of extrusion-aided protein fractionation optimization model

Annex 2. Pearson correlation of protein-rich fraction and starch-rich fraction properties

	P.content	P.yield	S.content	P.BD	S.BD	P.sol.P	P.sol.PS	P.sol.PSD	P.WAC	P.OAC	S.WAC
P.content	1	-,964**	,988**	,964**	,939**	-,941**	-,854**	-,387	,767*	-,849**	-,417
P.yield	-,964**	1	-,972**	-,873**	-,827**	,905**	,819**	,509	-,738*	,918**	,595
S.content	,988**	-,972**	1	,945**	,913**	-,958**	-,847**	-,346	,802**	-,832**	-,483
P.BD	,964**	-,873**	,945**	1	,995**	-,936**	-,852**	-,218	,780*	-,686*	-,245
S.BD	,939**	-,827**	,913**	,995**	1	-,914**	-,821**	-,171	,753*	-,630	-,169
P.sol.P	-,941**	,905**	-,958**	-,936**	-,914**	1	,825**	,274	-,867**	,724*	,417
P.sol.PS	-,854**	,819**	-,847**	-,852**	-,821**	,825**	1	,393	-,827**	,695*	,261
P.sol.PSD	-,387	,509	-,346	-,218	-,171	,274	,393	1	-,069	,713*	,213
P.WAC	,767*	-,738*	,802**	,780*	,753*	-,867**	-,827**	-,069	1	-,531	-,437
P.OAC	-,849**	,918**	-,832**	-,686*	-,630	,724*	,695*	,713*	-,531	1	,584
S.WAC	-,417	,595	-,483	-,245	-,169	,417	,261	,213	-,437	,584	1
S.OAC	,708*	-,730*	,649	,611	,586	-,658	-,495	-,519	,530	-,778*	-,538
P.RHC.BW	,892**	-,832**	,891**	,939**	,924**	-,896**	-,911**	-,222	,780*	-,613	-,279
P.RHC.CW	,687*	-,583	,740*	,792*	,799**	-,792*	-,648	,195	,764*	-,267	-,074
P.TA.BW	-,219	,054	-,141	-,324	-,356	,043	,231	-,399	-,101	,000	-,133
P.TA.CW	-,303	,213	-,380	-,346	-,357	,425	,095	-,455	-,397	,045	,058
P.visc.25	,097	-,037	,081	,130	,147	-,253	,081	,012	-,011	-,049	,002
P.visc.95	,831**	-,699*	,805**	,919**	,937**	-,820**	-,772*	-,214	,634	-,498	,108
P.visc.final	,921**	-,848**	,909**	,964**	,955**	-,912**	-,885**	-,287	,728*	-,652	-,212
S.visc.25	-,905**	,773*	-,873**	-,980**	-,991**	,874**	,793*	,098	-,705*	,573	,120
S.visc.95	-,940**	,991**	-,949**	-,842**	-,793*	,873**	,766*	,510	-,674*	,911**	,645
S.visc.final	-,955**	,971**	-,958**	-,882**	-,844**	,900**	,737*	,387	-,688*	,870**	,621

	S.OAC	P.RHC.BW	P.RHC.CW	P.TA.BW	P.TA.CW	P.visc.25	P.visc.95	P.visc.final	S.visc.25	S.visc.95	S.visc.final
P.content	,708*	,892**	,687*	-,219	-,303	,097	,831**	,921**	-,905**	-,940**	-,955**
P.yield	-,730*	-,832**	-,583	,054	,213	-,037	-,699*	-,848**	,773*	,991**	,971**
S.content	,649	,891**	,740*	-,141	-,380	,081	,805**	,909**	-,873**	-,949**	-,958**
P.BD	,611	,939**	,792*	-,324	-,346	,130	,919**	,964**	-,980**	-,842**	-,882**
S.BD	,586	,924**	,799**	-,356	-,357	,147	,937**	,955**	-,991**	-,793*	-,844**
P.sol.P	-,658	-,896**	-,792*	,043	,425	-,253	-,820**	-,912**	,874**	,873**	,900**
P.sol.PS	-,495	-,911**	-,648	,231	,095	,081	-,772*	-,885**	,793*	,766*	,737*
P.sol.PSD	-,519	-,222	,195	-,399	-,455	,012	-,214	-,287	,098	,510	,387
P.WAC	,530	,780*	,764*	-,101	-,397	-,011	,634	,728*	-,705*	-,674*	-,688*
P.OAC	-,778*	-,613	-,267	,000	,045	-,049	-,498	-,652	,573	,911**	,870**
S.WAC	-,538	-,279	-,074	-,133	,058	,002	,108	-,212	,120	,645	,621
S.OAC	1	,497	,125	-,069	,099	,277	,382	,527	-,541	-,735*	-,753*
P.RHC.BW	,497	1	,759*	-,290	-,193	,129	,851**	,985**	-,920**	-,814**	-,833**
P.RHC.CW	,125	,759*	1	-,133	-,698*	,016	,821**	,759*	-,780*	-,526	-,573
P.TA.BW	-,069	-,290	-,133	1	,004	,124	-,221	-,253	,448	,052	,151
P.TA.CW	,099	-,193	-,698*	,004	1	-,179	-,355	-,226	,350	,163	,252
P.visc.25	,277	,129	,016	,124	-,179	1	,089	,185	-,196	-,077	-,181
P.visc.95	,382	,851**	,821**	-,221	-,355	,089	1	,909**	-,921**	-,647	-,674*
P.visc.final	,527	,985**	,759*	-,253	-,226	,185	,909**	1	-,948**	-,829**	-,851**
S.visc.25	-,541	-,920**	-,780*	,448	,350	-,196	-,921**	-,948**	1	,746*	,812**
S.visc.95	-,735*	-,814**	-,526	,052	,163	-,077	-,647	-,829**	,746*	1	,983**
S.visc.final	-,753*	-,833**	-,573	,151	,252	-,181	-,674*	-,851**	,812**	,983**	1