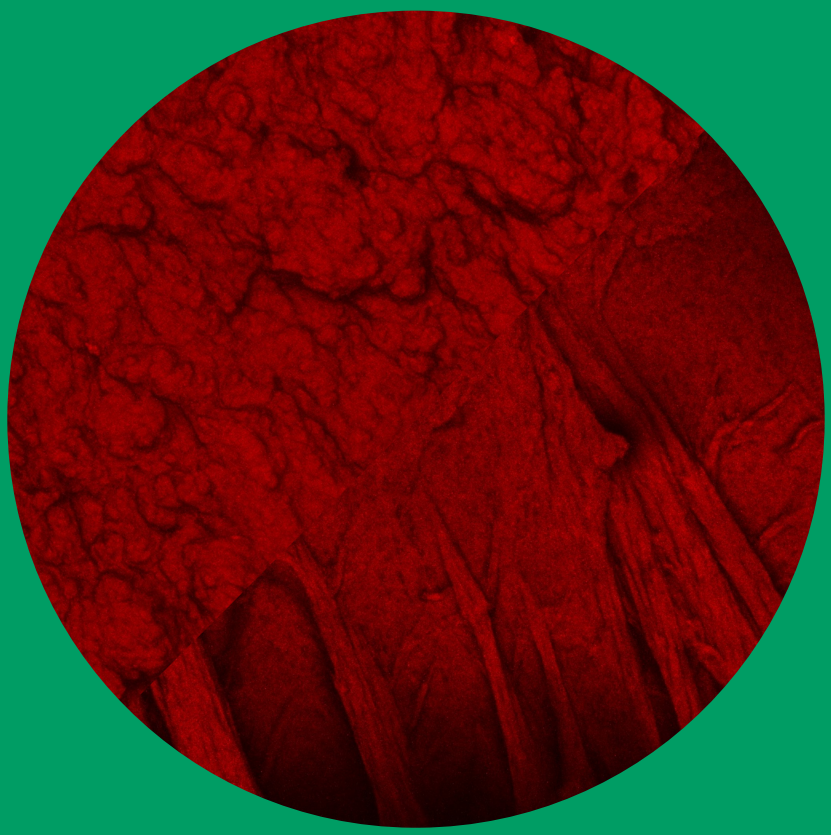
Department of Bioproducts and Biosystems

Functionalisation strategies for plant proteins in meat analogues and solubility-dependent food applications

Anni Nisov



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Functionalisation strategies for plant proteins in meat analogues and solubility-dependent food applications

Anni Nisov

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Abstract

This study aimed to enhance the functionality of plant proteins in food applications, focusing on solubility-dependent properties and fibrous structure formation in meat analogues produced by high-moisture extrusion processing. Plant protein ingredients are sustainable and healthy alternatives to their animal counterparts, but they often have limited functional properties. This research investigated various plant protein isolates and concentrates, including rice, pea, and wheat protein isolates, as well as pea, oat and rapeseed protein concentrates. These concentrates, produced through energy-efficient dry fractionation, also of-fer additional health benefits by increasing dietary fibre intake. Strategies to improve plant protein functionality included limited enzymatic protein hydrolysis to improve foaming, gelation, and colloidal stability of rice proteins, and chemical pH-shifting to acidic and neutral conditions to enhance fibrous structures of rice, pea, and wheat proteins. Different enzymatic crosslinking and deamidation treatments were applied to oat proteins, while acidification using fermentation was investigated to tackle sensory challenges in rapeseed ingredient. The findings of this study showed that limited hydrolysis improved functional properties of originally insoluble rice proteins, with a 1.5-1.8% degree of hydrolysis being generally preferable, especially for foaming and gelation. The choice of enzyme significantly influenced the outcomes, even with similar degrees of hydrolysis. The pH of the raw materials played a crucial role in fibrous structure formation during high-moisture extrusion processing. Neutral pH conditions enhanced structure formation in rice, wheat, and pea proteins due to the increased disulphide bond reactivity at higher pH levels, which was independent of the isoelectric point of the raw material. Successful fibrous structure formation in oat protein concentrate required a combination of pre-heating, deamidation, and cross-linking, highlighting the complex interplay between starch and proteins, as it was discovered that when starch was degrades, the fibrous structure formation was not induced. Furthermore, related to rapeseed, it was found that acidification, whether involving fermentation or incubation, negatively impacted fibrous structure formation, with different strains producing varied outcomes. While fermentation reduced unwanted chemical flavour and odour in rapeseed ingredient, it introduced proteolysis leading to intense bitterness and loss of fibrous structure. Chemical pH-shifting alone was removing the chemical flavour without introducing bitterness or loss in structure, suggesting it as a viable strategy for functionalisation in meat analogue applications. These results underscore the potential of the used strategies to accelerate the use of plant proteins in meeting the growing demand for sustainable and healthy food products. By using these strategies, this research contributes to the development of more appealing and sustainable plant-based alternatives, supporting both environment and consumer health.

Keywords Plant proteins, Fibrous structure, Enzymatic modification, pH-shifting, Fermentation

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Tiivistelmä

Tämän tutkimuksen tavoitteena oli parantaa kasviproteiinien toiminnallisia ominaisuuksia elintarvikesovelluksissa. Työ keskittyi proteiinien liukoisuudesta riippuvaisiin sovelluksiin ja liha-analogien säikeisten rakenteiden muodostumiseen korkean kosteuden ekstruusioprosessissa. Kasviproteiinit ovat ympäristöystävällisiä ja terveellisiä vaihtoehtoja eläinproteiineille, mutta niiden toiminnallisuus ruokasovelluksissa on rajallista. Tässä työssä tutkittiin riisi-, herne- ja vehnä-isolaatteja sekä herne-, kaura- ja rypsikonsentraatteja. Tutkitut proteiinikonsentraatit on tuotettu energiatehokkaalla kuivafraktioinnilla, mikä lisää ravintokuidun saantia ja siten tuo lisäterveyshyötyjä. Työn menetelmiin kuului rajoitettu entsymaattinen proteiinihydrolyysi, jolla parannettiin riisiproteiinien vaahtoutumista, geeliytymistä ja kolloidista stabiilisuutta. Lisäksi tutkittiin kemiallista pH-säätöä riisi-, herne-, ja vehnäproteiinien säikeisten rakenteiden parantamiseksi. Erilaisia entsymaattisia ristisilloitus- ja deamidaatiokäsittelyjä sovellettiin kauraproteiineihin, kun taas fermentoinnin avulla tapahtuvaa hapattamista tutkittiin rypsin aistinvaraisten ominaisuuksien parantamiseksi. Tulokset osoittivat, että kontrolloitu hydrolyysi paransi alun perin liukenemattomien riisiproteiinien toiminnallisia ominaisuuksia, ja 1.5-1.8 %:n hydrolyysiaste oli yleisesti ottaen toimivin erityisesti vaahtoutumisen kannalta. Kasviraaka-aineiden pH:lla oli keskeinen rooli kuitumaisten rakenteiden muodostumisessa korkean kosteuden ekstruusioprosessissa: neutraalit pH-olosuhteen paransivat rakenteen muodostumista disulfidisidosten reaktiivisuuden lisääntyessä korkeammassa pH:ssa. Onnistunut säikeisen rakenteen muodostuminen kauraproteiinikonsetraatilla vaati esikuumennuksen ja entsymaattisen deamidaation ja ristisilloittamisen yhdistelmän. Tulokset korostivat tärkkelyksen ja proteiinien monimutkaista vuorovaikutusta, sillä havaittiin, että kun tärkkelys hajosi, kuitumaisten rakenteiden muodostumista ei kauraraaka-aineella tapahtunut. Lisäksi rypsin osalta havaittiin, että hapattaminen, olipa kyseessä fermentaatio tai pelkkä näytteen inkubointi kemiallisen pH:n alentamisen jälkeen, vaikutti negatiivisesti kuitumaisten rakenteiden muodostumiseen. Lisäksi eri bakteerikannat tuottivat erilaisia tuloksia. Vaikka rypsikonsentraatin fermentaatio vähensi ei-toivottuja kemiallisia makuja ja hajuja, se aiheutti proteolyysiä, joka aiheutti rakenteen menetyksen lisäksi voimakasta karvasta makua. Pelkkä kemiallinen pH-muutos (ilman inkubointia) poisti kemiallisen maun rypsikonsentraatista aiheuttamatta karvautta tai rakenteen menetystä, mikä viittaa siihen, että se on käyttökelpoinen strategia lihankorvikkeiden säikeistämisessä. Tutkitut menetelmät voisivat lisätä kasviproteiinien käyttöä elintarvikkeissa, vastaten kestävien ja terveellisten ruokien kysyntään, mikä lisäisi kansanterveyttä ja ympäristöystävällisyyttä.

Avainsanat Kasviproteiinit, säikeinen rakenne, entsyymikäsittely, pH-säätö, fermentointi

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I wish to express my sincerest gratitude to my supervising professor, Alexander Frey, for his continuous guidance and support throughout this research journey. I would also like to extend my hearfelt thanks to my advisor, Dr. Emilia Nordlund, whose constant support has been a source of motivation and strength. I deeply admire her everlasting enthusiasm for food science and its future, her ability to think critically, and her remarkable ability to take determined action in any situation. Her mentorship has been truly ispiring to me for which I am forever grateful. I also want to acknowledge Professor Nesli Sözer, the final co-author on two of my Publications, for her invaluable guidance not only in the research but also in shaping the overall direction of my work.

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Espoo, 13 November 2024
Anni Nisov

Contents

List of Abbreviations and Symbols

HMEP	high-moisture extrusion processing
OPC	oat protein concentrate
PPC	pea protein concentrate
PPI	pea protein isolate
RP	rice endosperm protein isolate
RPC	rapeseed protein concentrate
WG	wheat gluten

List of Publications

This doctoral dissertation consists of a summary of the following publications, which are referred to in the text by their Roman numerals:

1. Nisov, Anni; Ercili-Cura, Dilek; Nordlund, Emilia. 2020. Limited hydrolysis of rice endosperm protein for improved techno-functional properties. Elsevier. Food Chemistry, volume 302, issue 125274. https://doi.org/10.1016/j.foodchem.2019.125274.

2. Nisov, Anni; Nikinmaa, Markus; Nordlund, Emilia; Sozer, Nesli. 2022. Effect of pH and temperature on fibrous structure formation of plant proteins during high-moisture extrusion processing. Elsevier. Food Research International, volume 156, issue 111089. https://doi.org/10.1016/j.foodres.2022.111089

3. Pöri, Pinja; Nisov, Anni; Nordlund, Emilia. 2022. Enzymatic modification of oat protein concentrate with trans- and protein-glutaminase for increased fibrous structure formation during high-moisture extrusion processing. Elsevier. LWT – Food Science and Technology, volume 156, issue 113035. https://doi.org/10.1016/j.lwt.2021.113035.

4. Nisov, Anni; Valtonen, Anniina; Aisala, Heikki; Spaccasassi, Andrea; Walser, Christoph; Dawid, Corinna; Sozer, Nesli. 2024. Effect of peptide formation during rapeseed fermentation on meat analogue structure and sensory properties at different pH conditions. Elsevier. Food Research International, volume 180, issue 114070. https://doi.org/10.1016/j.foodres.2024.114070

Author's Contribution

Publication 1: Limited hydrolysis of rice endosperm protein for improved techno-functional properties

AN participated in designing the work under the supervision of EN and DEC. AN was responsible for the hands-on execution of experimental work, calculations, and the interpretation of the results. Additionally, AN was responsible for drafting the initial version of the publication and finalising it with her co-authors. Notably, AN developed the surface hydrophobicity method by building upon existing literature. Furthermore, she introduced a novel approach for calculating the degree of hydrolysis, eliminating the necessity for acid hydrolysis during the analysis. She also introduced a novel approach to calculate colloidal stability results, also considering the presence of sedimented insoluble particles.

Publication 2: Effect of pH and temperature on fibrous structure formation of plant proteins during high-moisture extrusion processing

AN had the main responsibility in designing the publication entity under the supervision of EN and NS. She had the main responsibility in coordinating and executing the experimental part as well as in the data analysis. MN was responsible for the statistical analysis. AN planned and executed the high-moisture extrusion trials and coordinated the rest of the experimental work. Additionally, AN drafted the initial version of the publication and finalised it with the co-authors.

Publication 3: Enzymatic modification of oat protein concentrate with trans- and protein-glutaminase for increased fibrous structure formation during high-moisture extrusion processing

AN had the main responsibility in designing the publication under the supervision of EN. She had the main responsibility in coordinating the work and supervising PP through the execution of experimental work and data analysis. PP was responsible for drafting the original version of the publication, with AN significantly contributing the writing and modifying process, particularly in the discussion part.

Publication 4: Effect of peptide formation during rapeseed fermentation on meat analogue structure and sensory properties at different pH conditions.

AN was responsible for designing the publication entity under supervision of NS. AN responsibility included coordinating the work and collaborating with AS, CH, and CD from the Technical University of Munich regarding their responsibility of peptidomics analysis. AV was responsible for fermentation and HA for sensory analysis. AN executed the high-moisture extrusion processing and organised the remaining experimental work and data analysis. AN performed all other data analysis except for peptidomics and sensory results. AN drafted the original version of the publication and finalised it with the co-authors.

1. Introduction

Global warming and continuously increasing world population has drawn considerable attention to the use of plant proteins as alternative sources for animal counterparts. Environmental concerns surround the consumption of milk and meat products, particularly derived from livestock as it contributes to greenhouse gas emissions, high water usage, wide land use, deforestation, and release of methane (Mottet et al., 2017; Zhang et al., 2022a). Simultaneously, health-conscious consumers seek dietary options that align with their ethical, health and environmental values. In response to these concerns, plant-based proteins emerge as sustainable and nutritionally competitive alternatives (Saerens et al., 2021; Smetana et al., 2015).

According to the 2022 FAOSTAT data, the global production volumes for cereals, pulses, and oilseeds reached 3 060 Mt, 96 Mt, and 1143 Mt, respectively. Among the cereals, wheat was the second most cultivated crop with a production volume of 808 Mt) followed by paddy rice at 776 Mt. Oats, with a production volume of 26 Mt ranked as the seventh most cultivated cereal grain worldwide. In the category of pulses, peas ranked third with a worldwide production volume of 14 Mt. In the oilseeds category, rapeseed was the third most cultivated crop globally with a production volume of 87 Mt. However, only 55% of the calories produced by the world's crops are utilised for human consumption, while 36 % are used for feed, and the remaining 9 % is used as biofuels (Cassidy et al., 2013). Moreover, a review by Wouters et al. (2016) revealed that only 33% of plant proteins worldwide were used directly for food applications in 2011.

Regardless of the vast availability of cultivated plant crops, the challenge in utilising extracted plant proteins lies in their limited functional properties. These include solubility, foaming, gelation, emulsification, colloidal stability, and fibrous structure formation, which are often inferior when compared to animal proteins (Day et al., 2022). For instance, egg white serves an excellent foaming, gelling and structure-stabilising agent in various food applications such as puddings, cakes, meringues, and cookies (Razi et al., 2023). The superior performance of these properties is attributed to the high solubility (>95%) of egg white proteins in their undenatured state. In contrast, commercial plant-proteins often exhibit less than 40% protein solubility, leading to limited performance in such applications. Another example of superior animal-derived proteins includes casein proteins, which maintain colloidal stability in aqueous solutions, forming milk. This ability is particularly important in beverage, cheese, and yoghurt applications. The uniqueness of casein is attributed to its lack of disulphide bond formation, leading to disordered, open, and flexible protein structure, resulting in colloidal system known as casein micelle (Day et al., 2022). The lack of colloidal stability of plant proteins is often attributed to their distinct aggregation via disulphide bond formation leading to limited functionality. Further examples of superior animal-derived proteins include meat proteins, such as actin and myosin, originating from muscle tissue, exhibit

a fibrous structure that contributes to the texture and mouthfeel characteristic of meat products. The uniqueness of actin and myosin lies in their ability to form long protein chains called myofibrils packed into a muscle cell (Koubassova and Tsaturyan, 2011). In contrast, plant-proteins are often globular-shaped or irregularly-shaped particles tightly aggregated to each other after their extraction leading to limited fibrous structure formation without any further processing.

The protein content in cereals, legumes, and oilseeds ranges from approximately 10% to 40% (Shewry et al., 1995). while the protein content in egg white (Abeyrathne et al., 2013) and red meat (Williams, 2007) can exceed 80% when measured on dry weight basis. Consequently, plant proteins often require an isolation or concentration process to achieve similar protein concentration levels as their animal-based counterparts.

The most commonly used commercial method for protein isolation, referred as wet extraction, involves several steps, including alkaline solubilisation, isoelectric precipitation, and drying. This process can yield protein concentration levels of 75-95%. However, one critical drawback of this approach is that these processing steps cause proteins to unfold from their native state, leading to substantial protein denaturation and aggregation, which diminishes their applicability especially in solubility-dependent food applications. Furthermore, the energy-intensive nature of these steps results in increased energy demand, leading to a higher environmental impact. Another commercially available method for protein enrichment is known as dry fractionation. This method relies on milling and subsequent physical separation of protein-rich fractions from starch, or fibre-rich fractions based on their particle size, shape, and density eliminating the need for water and chemicals (Assatory et al., 2019). It has been widely confirmed that dry fractionation produces proteins in their native state (Pelgrom et al., 2015) and has a lower environmental impact compared to wet extraction (Assatory et al., 2019; Lie-Piang et al., 2021; Shanmugam et al., 2023). However, dry fractionation also has its limitations, such as achieving considerably lower protein concentration ranging from 28 to 70% (Assatory et al., 2019; Schutyser et al., 2015), when mass yields are reasonable (Assatory et al., 2019).

Given the challenges and limitations associated with the use of plant proteins, this study aimed to enhance their functionality for improved utility in food applications. The focus was on two main areas: improving the functionality of wet-extracted raw materials with high protein content for solubility-dependent applications and developing strategies to enhance the fibrous structure formation of plant proteins in meat analogue applications. Given the excessive protein consumption and insufficient fibre intake in Western diets, and the fact that most meat alternative studies have primarily utilised highly purified soy, wheat, and pea-based protein isolates obtained through wet fractionation, this study also aimed to identify functionalisation strategies applicable for dry fractionated low-protein raw materials. These materials often fail to form strong fibrous structures and prominent protein alignment due to their low ratio of protein. The studied functionalisation strategies included limited enzymatic hydrolysis for improved foaming, colloidal stability, and gelation properties. In addition, crosslinking enzymes, fermentation, and pH-shift were utilised as tools to enhance the fibrous structure formation ability of plant proteins in high-moisture extrusion processing, a technique commonly used to generate meat analogues.

2. Background

2.1 Plants as a protein source

Proteins typically exhibit a hierarchical organisation involving three to four levels of structural complexity. The primary structure denotes the linear sequence of amino acids. The secondary structures, such as a-helices, beta-turns, beta-sheets, and random coils emerge through non-covalent interactions between the side chains of the amino acids (hydrophobic interactions, electrostatic attraction, hydrogen bonds, and Van der Waals forces). Tertiary structures evolve when both covalent disulphide bridges and additional non-covalent interactions form to provide stability to the protein structure. Lastly, quaternary protein structures arise when different protein subunits, previously organised as tertiary structures, combine to form functional protein complexes through a blend of non-covalent and covalent interaction. For example, several plant storage proteins possess a compact structure composed of multiple subunits linked to each other through covalent and non-covalent interactions. (Jiang et al., 2018; Shewry et al., 1995). This background section provides an overview of selected plant proteins, detailing the chemical compositions of different plant proteins sources, outlining their protein classes, and introducing relevant protein extraction methods.

2.1.1 Protein composition and structure

This study focused on five distinct plant protein sources: rice, wheat, oats, peas, and rapeseed. Table 1 presents the chemical composition of the seeds of these plant protein sources. Additionally, Table 1 presents the composition of rapeseed meal, the residue after oil-pressing, as in that way the lipid levels are more comparable to the whole cereal and legume seeds. On average, cereals, such as rice, oats, and wheat exhibit the lowest protein content, ranging from 4-18%, 8-17%, and 6-27%, respectively (Arendt and Zannini, 2013; Sterna et al., 2016). Legumes and oilseeds, such as peas and rapeseed display significantly higher average protein contents, ranging from 21-24% for peas and 20-25% for rapeseed (Eriksson et al., 1994; Nadathur et al., 2017). Once the oil is pressed out from rapeseed seeds the protein ratio in the residual meal ranges from 34-40% (Rommi et al., 2014). Starch content is notably higher in rice (77%) and wheat kernels (61-75%), whereas oats (40-50%) and peas (42-46%) exhibit a lower amount of starch (Amagliani et al., 2017; Arendt and Zannini, 2013; Nadathur et al., 2017; Šramková et al., 2009). In contrast, rapeseed meal contains negligible levels of starch, ranging from 0.1% (specifically studied in cotyledons) to 3.0% (comprising the entire rapeseed meal) as reported by Blair and Reichert (1984) and Rommi et al. (2014), respectively. Although there is limited research

on the starch content of whole rapeseed seeds, one can conclude that the starch content is lower in the intact full-fat seed than in the oil-pressed meal. Cereals and legumes exhibit relatively low fat contents, typically ranging from 2-8%, while oilseeds, specifically rapeseed, can contain as much as 44% fat (Table 1). While rice exhibits a relatively low dietary fibre content (1-3%), oats (11-18%) and wheat (12-16%) contain higher amounts (Amagliani et al., 2017; Andersson et al., 2013; Belitz et al., 2009; Sterna et al., 2016). Peas stand out with a notable fibre content of 21% (Table 1). Rapeseed seeds exhibit a fibre content between 16-20%, and in rapeseed meal, this content increases up to 32% (Eriksson et al., 1994; Nadathur et al., 2017). The ash content in rice, wheat, oats, and peas falls within a similar range (2-3%). In contrast, rapeseed seeds exhibit a higher ash content (4%), which further accumulates in the residual meal after oil-pressing, resulting in an increased content of 7% (Table 1).

Table 1. Average chemical composition (%, dry matter basis) in whole seeds of rice, wheat, oats, peas, rapeseed, and oil-pressed rapeseed meal. Average values are calculated from the references listed in this caption.

Seed	Protein	Fat	Fibre	Ash	Starch
Rice [a]	8.9	2.6	1.1	1.5	76.7
Wheat [b]	13.5	2.5	15.3	1.7	53.3 [Ω]
Oat [b]	14.9	8.2	11.1	3.3	52.9 [Ω]
Pea [c]	22.5	1.8	21.2 [Φ]	2.1	44.0
Rapeseed [d]	22.9	43.8	16.9 [X]	4.2	na
Rapeseed meal [e]	35.9	11.8	32.3 [c]	7.0	0.2

[Φ] Data was available only on a “as is” basis
[X] Sum of analysed dietary fibre polysaccharide residues, uronic acid, and Klason lignin
[Ω] Estimated as available carbohydrates minus fibre

Abbreviations: na = not available

References: [a] Amagliani et al. (2017), [b] Belitz et al. (2009), [c] Nadathur et al. (2017), [d] Eriksson et al. (1994), [e] Rommi et al. (2014)

When applying plant protein ingredients in food manufacturing, it is equally crucial to consider both the protein content and its quality to be able to tailor it to specific purposes. For example, the production of meat analogues demands proteins with lower solubility, whereas applications like beverage, pudding, and meringues benefit from highly soluble proteins. It is also crucial to note that the latter applications require functional properties such as gelation and foaming, which in turn are not only dependent on solubility but also the size of the protein. Table 2 presents the protein fractions of rice, oats, wheat, peas, and rapeseed according to their solubility in water (albumin), dilute saline solution (globulin), alcohol (prolamin), and dilute acid or alkali (glutelin), known the Osborne fractions (Osborne, 1897), as well as the protein size in these fractions.

In milled rice, the major protein fraction is glutelin, accounting for 75-81% of total proteins, followed by globulin (6-13%), albumin (4-6%), and prolamins (2-7%) (Amagliani et al., 2017). Glutelin is a high molecular weight storage protein comprising of α (acidic) and β (basic) subunits with molecular weights of 30-40 and 19-23 kDa, respectively. Initially, glutelin is synthetised as a polypeptide with a molecular weight ranging from 51-57 kDa and is subsequently enzymatically hydrolysed into α and β subunits that are further polymerised into glutelin macromolecule through disulphide bonding. In oats, the most abundant protein group is globulin (50-80% of total proteins), followed by prolamin (4-14%), albumin (1-12%), and glutelin (<10%)(Klose and Arendt, 2012). The major component of globulins in oats is 12 S storage protein, with a size range of 53-58 kDa, comprising

α (acidic) and β (basic) subunits having sizes of 32-37 and 22-24 kDa, respectively. Wheat proteins are typically classified into gluten (glutelin and prolamin) and non-gluten proteins (albumin and globulin), which make up 80 and 10% of total proteins, respectively (Gammoh et al., 2023; Schalk et al., 2017). The remaining 10% is unfractionated insoluble residue. Gluten proteins are further categorised into monomeric gliadins (prolamin fraction) and polymeric glutenins (glutelin fraction) accounting for 53% and 26% of total proteins, respectively. Prolamins are subdivided into ω5- (51 kDa), ω1,2- (43 kDa), α- (32 kDa) and γ-gliadins (35 kDa), while glutelins are subdivided into low molecular weight glutenins (32 kDa) and high molecular weight glutenins (69 and 87 kDa). In peas, the two predominant protein groups are globulin (65-80%) and albumin (10-20%)(Shanthakumar et al., 2022). Globulins serve as the primary storage proteins and can be further categorised as vicilin (7S), legumin (11S) and convicilin, with molecular sizes of 47-50 kDa, 60-80 kDa and 70 kDa, respectively. The rapeseed proteins consist of 10-21% 2s napin (albumins), 60-70% 11S/12S cruciferin (globulin), and 8-20% oleosins (prolamins) corresponding to sizes of 12.5-14.5 kDa, 41-55 kDa, and 19 kDa, respectively (Deleu et al., 2010; Murphy et al., 1991; Tan et al., 2011; Wanasundara et al., 2017).

Table 2. Protein fraction and size in white rice, oats, wheat, peas, and rapeseed according to Osborne classification where albumins are soluble in water, globulins in dilute saline solution, prolamins in alcohol, and glutelins in dilute acid or alkali.

Raw material	Albumin		Globulin		Prolamin		Glutelin	
	%	Size (kDa)	%	Size (kDa)	%	Size (kDa)	%	Size (kDa)
Rice [a]	4–6	10-150 - 40, 55, 60 (major subunits)	6-13	19–22 (γ-globulin) 21 (α-globulin) 53-56, 16, 25	2–7	13 (predominant) 10 16	75-81	30-40 (α-glutelin) 19-23 (β-glutelin) 52 (glutelin precursor)
Oats [b]	1–12	14-17 20-27 36-47 (2S albumins)	50-80	53-58 (12S storage) - 32-37 (α-polypeptide) - 22-24 (β-polypeptide) 50-70 (7S storage) 48-52 (3S storage)	4–15	17-34 (avenins)	<10	Unextracted globulins and prolamins, minor polypeptides group
Wheat [c]	11	56, 39, 31, 21, 10, 9, 8 (combined fraction of albumins and globulins)			53	Gliadins: 51 (ω5) 43 (ω1,2) 32 (α) 35 (γ)	26	Glutenins: - 32 (LMW-GS) - 69 (γ, HMW-GS) - 87 (x, HMW-GS)
Peas [d]	10–21	5-80 (2S pea albumin 1 and pea albumin 2)	65-80	47-50 (7S, vicilin) 60-80 (11S, legumin) 70 (7S, convicilin)	4-5	Insoluble fraction	3-4	na
Rapeseed [e]	10-20	13-15, 14 (2S napin) - ~4.5 - ~10	60-70	300 (11S, cruciferin, formed of six subunits) - 41-55 - 30 (α-chain) - 20 (β-chain)	8-20	19 (oleosins) 30 (caleosins) trypsin inhibitor	nd	na

Abbreviations: LMW-GS = low-molecular-weight glutenin subunits, HMW-GS = high-molecular-weight glutenin subunits, na = not available

References: [a] Amagliani et al. (2017), [b] Klose and Arendt (2012), [c] Gammoh et al. (2023); Schalk et al. (2017), [d] Shanthakumar et al. (2022), [e] Deleu et al. (2010); Murphy et al. (1991); Wanasundara et al. (2017)

2.1.2 Production of protein isolates and concentrates

The most commonly employed protein purification method for producing commercial protein isolates is the alkaline extraction process with subsequent isoelectric precipitation (Hernández-Álvarez et al., 2023). Alkaline extraction is usually the preferred approach for generating rice, oat, pea, and rapeseed protein isolates, while the industrial extraction of the major wheat protein, gluten, follows a method exclusive to wheat (Figure 1). In sum-

mary, the alkaline extraction method involves the solubilisation of proteins under alkaline conditions, followed by centrifugation and subsequent precipitation of the supernatant at the isoelectric point of the protein. The resulting sediment can be further neutralised and finally dried using various drying methods. The extraction of gluten relies on its specific properties, namely the formation of a special protein network and its insolubility in water (Ortolan and Steel, 2017). After the wheat grains have been milled, they are soaked in water, initiating the dough development process. This dough development process enables the separation of proteins from starch and water-soluble components through a water-washing step. After washing, the gluten fraction is dried and milled, while the starch fraction is directed to the starch production process.

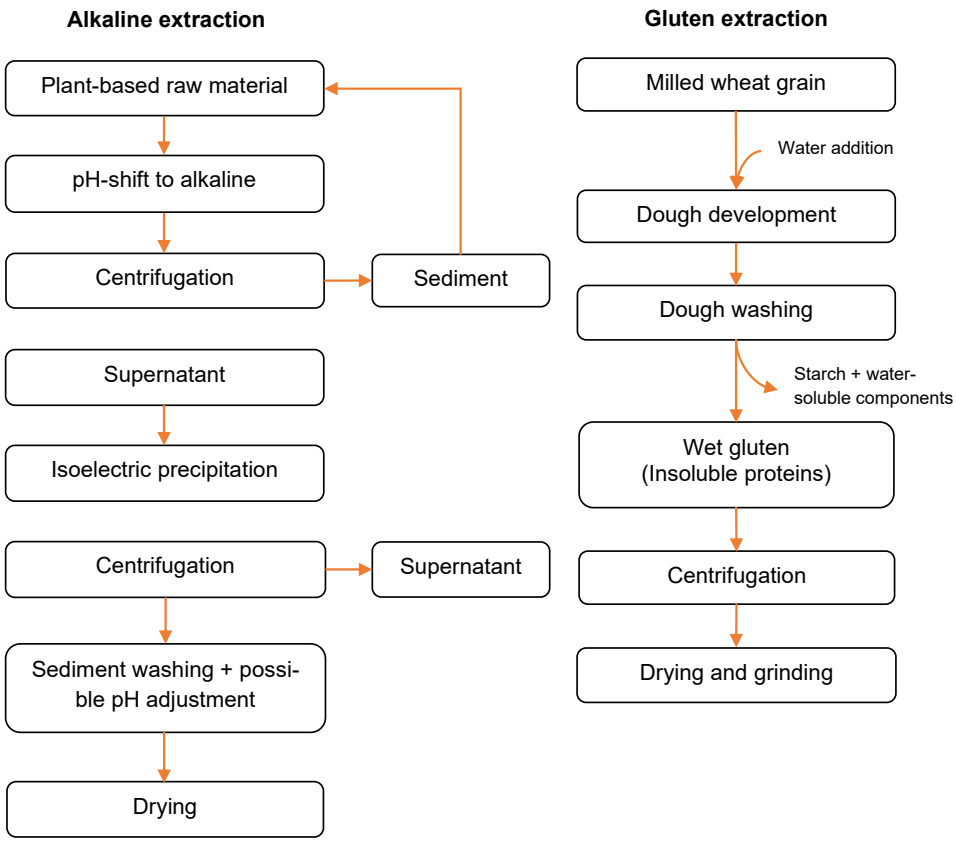


Figure 1. Schematic illustrations of two most commonly used wet extraction methods for producing protein isolates: alkaline extraction and gluten extraction.

An alternative commercially adopted technique by food ingredient industry for protein enrichment is dry fractionation. This method generates protein concentrates and includes steps such as dry milling and air classification (Figure 2). The advantage of this approach lies in its sustainability and cost-effectiveness, as it eliminates the need for the addition of water and solvents during processing. There are also other methods referred to as dry fractionation methods, such as triboelectroseparation and electro-spinning; however, they are not currently available at industrial scale.

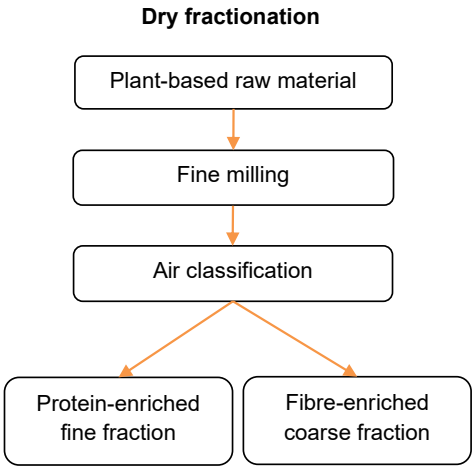


Figure 2. Commercially available air-classification based on dry fractionation method for protein concentrate production.

Dry fractionation has a significantly lower environmental impact across all categories, from water consumption to global warming potential, compared to wet fractionation (Lie-Piang et al., 2021). The latter consumes a high amount of water, chemicals, and energy. Commercially, dry fractionated ingredients derived from legumes exhibit protein concentrations ranging from 55-64% (De Angelis et al., 2024). On the other hand, wet fractionated protein isolates, such as those derived from wheat and soy, demonstrate notably higher protein contents, varying from 70-90% (Deng et al., 2023; Liu et al., 2021; Wang et al., 2006).

Table 3 (in the next section) presents existing research on proteins fractionated from various sources, including wheat, rapeseed, peas, and oats, using both wet and dry fractionation processes. An important factor influencing the protein concentration, in addition to process parameters, is the protein source and its specific variety. The protein concentration of dry fractionated ingredients can range from 20% to 56% when the mass yield is kept reasonable, depending on the plant type and process parameters (De Angelis et al., 2020; Silventoinen et al., 2019). Cereals such as rice, oats, and wheat exhibit lower protein concentration values of 20%, 23%, and 31% respectively, while legumes, such as peas (56%), and oilseeds, such as rapeseed (42%) exhibit higher protein concentration. In contrast, wet fractionation using alkaline extraction can result in protein concentration values ranging from 80 % to 95% (Chandi and Sogi, 2007; Shevkani et al., 2015). Two reported exceptions for rice and rapeseed resulted in notably lower protein contents of only 52-71% under notably milder process conditions. For rice, this involved non-heated alkaline solubilisation at a lower pH of 9 and isoelectric precipitation under cold conditions at 4 °C. In contrast, other rice-related studies used solubilisation temperatures from 40 to 55 °C using pH 10-11, with isoelectric precipitation conducted at room temperature leading to protein concentrations between 85-88%. In addition, wet extraction of gluten can also result in protein concentration values between 72% and 82% (Liu et al., 2021; Ortolan and Steel, 2017; Wang et al., 2006).

In conclusion, the main differences between the two fractionation methods lie in their environmental impact, and economic viability. Dry fractionation has a lower environmental footprint, but it also results in lower protein concentration compared to wet fractiona-

tion. Wet fractionation can produce high-concentration protein isolates, but with a higher cost and burden to the environment. Therefore, there is a trade-off between the sustainability and the protein concentration of the plant protein ingredients produced by these methods. Moreover, the selection of protein extraction method has a major impact on the protein functionality, which is discussed in the next section.

2.1.3 Protein properties through different extraction methods

When assessing the suitability of plant proteins in food applications, it is important to understand the key functional properties. These include solubility, foaming, emulsifying, gelation, and colloidal stability as well as water- and oil-holding capacities. Table 3 provides a summary of some of these properties, with a specific focus on proteins derived from rice, wheat, peas, and oats using both dry and wet fractionation techniques.

The functional properties of plant-derived proteins obtained through alkaline extraction are influenced by several factors, including the process parameters, variety of the seed and the nature of the proteins in the starting raw material. For instance, when considering rice, different results can be obtained depending on whether the starting raw material is bran, which is rich in water-soluble proteins (albumins) or endosperm, which contains the water-insoluble proteins (glutelins). Studies that extracted proteins from rice endosperm reported low protein solubilities under 15% (Paraman et al., 2008; Zhao et al., 2012), while studies using rice bran achieved notably higher protein solubility values ranging from 47% to 80% (Bandyopadhyay et al., 2008; Chandi and Sogi, 2007).

In the case of rapeseed, Chabanon et al. (2007) reported that following isoelectric precipitation, the precipitate primarily consists of insoluble globulins, with albumins remaining in the supernatant. Consequently, if only the precipitated proteins are included in the final ingredient, the resulting solubility is relatively low, ranging from 10 to 26%, depending on the final pH (Chabanon et al., 2007; Yoshie-Stark et al., 2008). However, if the precipitation step is bypassed and all proteins in the supernatant are included in the ingredient, the resulting solubility for rapeseed proteins can increase as high as 75% (Jia et al., 2021b).

Considering oat proteins, all the studies listed in Table 3 exhibited relatively low solubility, ranging from 10% to 26%. This lower solubility can be attributed to the composition of protein fractions in oats, which may account to almost 80% of globulin (insoluble only in saline water), while the proportion of water-soluble albumin fraction may be as low as 1-12%. On the other hand, peas, which can also contain up to 80% globulins and 10-20% albumins, resulted in protein solubility between 50-80% under laboratory extraction conditions, depending on the cultivar and process parameters (Cui et al., 2020; Shevkani et al., 2015). Interestingly, commercial pea proteins exhibit notably lower protein solubility values, ranging from 12% to 27%. This implies that the processing at an industrial scale may be more severe, leading to significant protein denaturation, aggregation and reduced solubility (Lie-Piang et al., 2023). Additionally, various commercial wheat gluten ingredients have been found to exhibit nearly zero solubility in water (<5%) at pH 7 (Liu et al., 2021; Wang et al., 2006). In accordance with the low protein solubility, gluten samples exhibited low emulsion activities (0.2-5 m^2/g) and poor foaming capacities at around 30%.

The final pH of the raw material is a significant process parameter that influences the resulting functionality of proteins. Solubility, often seen as a prerequisite for other functional properties, is highly dependent on pH (Day, 2013). Therefore, the functional properties are also significantly affected by pH. For example, considering the alkaline-extracted rice

endosperm protein, after isoelectric precipitation, the final pH was adjusted to 7. Despite its near-zero solubility at this pH, it demonstrated a foaming capacity of 116% (Zhao et al., 2012). On the other hand, proteins extracted from rice bran were maintained at a pH of 4. Despite their higher solubility across a pH range from 3 to 9 (with solubility values between 29% and 85%), these proteins achieved a foaming capacity of only 70% (Bandyopadhyay et al., 2008). When emulsifying activity was measured at pH 8, the rice-bran derived proteins exhibited a value of 109 m^2/g, which was significantly higher than the nearly insoluble endosperm proteins that displayed only an activity of 4.2 m^2/g at pH 7.

Similarly, regarding oat proteins, it was observed that higher emulsion activities (35-49 m2/g) were associated with slightly higher protein solubility ranging from 18-26% (Mirmoghtadaie et al., 2009; Yue et al., 2021), while the lower emulsion activity of 12.5 m^2/g corresponded to reduced solubility of 14% (Cheng et al., 2022). These examples highlight the complex interactions between pH, solubility, and the functional properties of proteins. On the other hand, the low protein solubility reported by Cheng et al. (2022) was associated with higher water and oil-holding capacities, in contrast to the notably lower values reported by Mirmoghtadaie et al. (2009), who had higher protein solubility.

Additionally, the functionality can vary across different species or cultivars within the specific plant source. For instance, protein solubility values ranged from 47-73% across rice protein isolates derived from brans from three different cultivars (Chandi and Sogi, 2007). Protein isolates derived from four different yellow pea cultivars resulted in emulsion stabilities of 2-37 min and foam capacities of 220-250% (Cui et al., 2020). Protein isolates from five field pea cultivars led to emulsion activity indexes of 1-14 m^2/g, corresponding to stabilities of 52-59 min (Shevkani et al., 2015). Lastly, oat protein isolates derived from four different cultivars showed foam capacities ranging from 125-200% (Yue et al., 2021).

While functional properties of dry fractionated plant proteins are less studied, dry fractionation has the advantage of preserving proteins in their native state, often leading to enhanced protein solubility and functionality (Pelgrom et al., 2015). For instance, wheat bran-derived protein-rich fractions showed a higher solubility (45%) compared to the nearly insoluble commercial gluten ingredients (Silventoinen et al., 2021). Similarly, dry fractionated rapeseed protein exhibited a solubility of 45% (Wockenfuss et al., 2023), higher than the 10-26% achieved through wet fractionation using alkaline and isoelectric precipitation. However, pea protein ingredients derived through dry fractionation exhibited surprisingly low protein solubility values ranging from 15-25% (De Angelis et al., 2021; Do Carmo et al., 2020), similar to commercial pea protein isolates (12-27%). Rice bran proteins enriched through dry fractionation demonstrated high protein solubility between 60%-70% (Silventoinen et al., 2019), comparable to those obtained through alkaline extraction in laboratory conditions.

In summary, the protein properties after dry fractionation and wet fractionation can differ significantly depending on plant source, variety, protein type, and process conditions but dry fractionation is able to preserve the protein in its native state while wet fractionation, particularly in industrial scale, can lead to protein denaturation and aggregation.

Table 3. Protein content, solubility and other functional properties of rice, wheat, rapeseed, pea, and oat proteins after wet and dry fractionation. Wet fractionation includes alkaline solubilisation and subsequent isoelectric precipitation while dry fractionation includes dry milling and air classification. The arrow (→) stands for pH adjustment and drying after isoelectric precipitation (if any).

Raw material	Protein extraction/enrichment method	Protein (%)	Solubility (%)	EAI/EC m^2/g	ES (min)	FC (%)	FS (%)	WHC (g/g)	OHC (g/g)
Rice flour [a]	Alkaline; pH 11.0 (3 h, 40 °C) + IEP; pH 4.5 (1 h, 4 °C) → pH 7.0, freeze-dried	84.7	15	1.7 (A_{500})	16	na	na	na	na
Rice endosperm flour [b] Defatted	Alkaline; pH 11.0 (4 h, 40 °C) + IEP; pH 4.5 (1 h, 4 °C) → pH 7.0, freeze-dried	87.9	<5	4.2	31% (10 min)	116	98 (20 min)	2.8	2.1
Rice bran [c] (3 different cultivars) Defatted; petroleum ether 3 different cultivars	Alkaline; pH 9.0 (1 h) + IEP; pH 4.5 (overnight, 4 °C) → pH 7.0, freeze-dried	52.5-58.9	47-73	40%	1 day	5-9	1.7-4.0 h (half-time in hour)	3.9-5.6	3.7-9.2
Rice bran [d] Sieved, defatted; hexane	Alkaline; pH 10, (1 h 50-55 °C) + IEP; pH 4.0 (1 h 50-55°C) → vacuum oven dried	86.2	80	109 (pH 8)	37	70 (pH 4)	84, 76 (10, 30 min)	na	na
Rice bran [e] Defatted; SC-CO2	Pin disc milling + Air classification	19.7-27.4	~60-70 (pH 6.8)	na	na	na	na	na	na
Wheat gluten [f] Commercial	Not disclosed	na	~10 (pH 8)	0.35 (A_{500})	~50% (30 min)	84 (pH nd)	40, 20 (10, 30 min)	na	na
Wheat gluten [g] Commercial	Not disclosed	71.5	~5	~5	9	29 (pH nd)	65, 30 (10, 30 min)	na	na
Wheat gluten [h] Commercial	Not disclosed	75.1	4.6	0.2 (pH nd)	80% (15 min)	~30 (pH nd)	80 (10 min)	na	na
Wheat bran [i]	Pin disc milling + Air classification	30.9	45 (pH 6.7)	na	na	na	na	1.2	1.0
Rapeseed flakes [j] Defatted; hexane	Alkaline; pH 7.4 + IEP; pH 5.8 → pH 7, pasteurised, spray dried	70.8	26	400 (mL/g)	85 %	na	na	na	na
Rapeseed meal [k] Defatted and ethanol-washed	Alkaline; pH n.d. (1h, RT) + IEP; pH 4.5 → no pH adjustment, freeze-dried	91.4	10 (pH 4.5)	18	30 %	44	10 % (30 min)	na	ns
Rapeseed presscake [l] Cold-pressed, defatted; petroleum ether	Alkaline; pH 8 (1 h 25 °C) + no IEP → freeze-dried	94.4	~75 (pH ~8)	na	na	na	na	~3	na
Rapeseed presscake [l] Cold-pressed, Defatted; hexane	Aqueous ethanolic washing, 75 °C + ZPS milling wit classifier wheel + sieving	61.9	~20	na	na	na	na	~5.6	na
Rapeseed presscake [m] **C**old-pressed, defatted; SC-CO2	UPZ milling + Air classification	42 %	~45 (pH 6.2-6.3)	na	na	na	na	na	na

Raw material	Protein extraction/enrichment method	Protein (%)	Solubility (%)	EAI / EC m^2/g	ES (min)	FC (%)	FS (%)	WHC (g/g)	OHC (g/g)
Yellow pea [n] (4 different cultivars) Dehulled, milled	Alkaline; pH 8.5-10 (1 h, RT) + IEP; pH 4.5 → pH 7.0, spray drying (130/80 °C)	80.4-85.7	50-80	na	2-37	250-220	50-95	na	na
Field pea [o] (5 different cultivars) Defatted; hexane	Alkaline; pH 9.0, (1 h, RT) +IEP; pH 4.5 → neutral pH, freeze-dried	90.8-94.7	64-80	1-14	52-95	87-132	94-96 (30 min)	3.9-4.8	5.5-7.2
Pea protein isolate [p] Commercial (3 different)	Not disclosed	83.2-87.3	~12-27	395-870 (ml/g pH 7.4-7.5)	na	na	na	2.5-5.4	1.3-1.7
Green pea [q] Dehulled	Micronisation + air classification	56	25 (pH nd)	na	na	na	na	0.76	0.51
Yellow pea [r] Dehulled	Milling + Air Classification	46.2	~25	17.2 (pH nd)	13 min	56 (pH nd)	na	0.97	1.14
Yellow pea [r] Whole	Milling + Air Classification	44	~15	18.8 (pH nd)	14 min	55 (pH nd)	na	0.93	1.12
Oat flour [s] Defatted; SC-CO2	Alkaline; pH 10 + IEP; pH 5.5, freeze-dried	92.2	10-20%	na	na	na	na	na	na
Oat flour [t] Defatted	Alkaline; pH 10, (1.5 h 40 °C) + IEP; pH 4.3 → pH 7, freeze-dried	90.9	~14	12.5	~28 min	~80	~40	~3.2	~2.3
Oat flour [u] Dehulled, defatted; hexane	Alkaline; pH 9.8, (1 h, RT) + IEP; pH 5.7 → pH 6.7-7	na	23	49	63 min	~800	~90 (30 min)	1.3	1.7
Oat flour [v] (4 different varieties) Dehulled defatted; hexane	Alkaline; pH 9.5 (1 h, RT) + IEP; pH 4.5 → pH 7, freeze-dried	86.9-89.4	~18-26	35-45	35-40 min	125-200	50-60 (30 min)	na	na
Non-heat treated oats [w] Dehulled, defatted; SC-CO2	Fine impact milling + Air classification	23.3-73.0	na	na	na	na	na	na	na

Abbreviations: IEP = isoelectric precipitation, EAI = emulsification activity index, EC = emulsion capacity, ES = emulsification stability index, FC = Paraman 2008 foam capacity, FS = foam stability, WHC = water-holding capacity, OHC = oil-holding capacity, and RT = room temperature, na = not available. All values for functional properties are expressed at pH 7 unless otherwise indicated. Some of the values have been derived from graphical data and, as such, may not be precise.

References: [a] Paraman et al. (2008), [b] Zhao et al. (2012), [c] Chandi and Sogi, (2007), [d] Bandyopadhyay et al. (2008), [e] Silventoinen et al. (2019) [f] Kong et al. (2007), [g] Wang et al., (2006), [h] Liu et al. (2021), [i] Silventoinen et al. (2021), [j] Yoshie-Stark et al. (2008), [k] Chabanon et al. (2007), [l] Jia et al. (2021b), [m] Wockenfuss et al. (2023), [n] Cui et al. (2020), [o] Shevkani et al. (2015), [p] Osen et al. (2014), [q] De Angelis et al. (2020), [r] Do Carmo et al. (2021), [s] Nivala et al. (2017), [t] Cheng et al. (2022), [u] Mirmoghtadaie et al. (2009), [v] Yue et al. (2021), [w] Sibakov et al. (2011)

2.2 Modification of plant-proteins for improved performance

The majority of plant proteins possess a compact structure, composed of numerous subunits interconnected through disulphide bonds, as well as hydrophobic and electrostatic interactions (Section 2.1.1). This structural complexity limits their functionality, thereby affecting their suitability in several food applications (Jiang et al., 2018). Therefore, to match the properties of animal-based proteins, it is crucial to explore different functionalisation strategies for improved performance. This section provides an overview of selected functionalisation techniques, including chemical pH-shift, enzymatic modification, and fermentation.

2.2.1 Chemical pH-shift

Section 2.1.3 showed that pH conditions are strongly linked to protein solubility, which in turn impacts various functional properties of proteins. The functionality of proteins is also inter-connected with other physicochemical attributes of proteins, such as its surface hydrophobicity, electrostatic repulsion, and particle size (Liang and Tang, 2013). At certain pH conditions, known as isoelectric point, the net charge of the proteins is zero, resulting in reduced solubility. Therefore, plant proteins typically display a U-shaped solubility curve, with the lowest solubility corresponding to their isoelectric point. Typically, plant proteins possess their isoelectric points at more acidic pH of 4-5.5 (Do Carmo et al., 2020; Liang and Tang, 2013; Nivala et al., 2017). However, wheat proteins have their isoelectric point at neutral pH of 7 (Kong et al., 2007). Given the strong relation between solubility and pH, pH-shifting has been studied as a promising functionalisation strategy to improve and modify the properties of plant-proteins, including solubility (Jiang et al., 2018).

The application of pH-shifting as a processing technique leads to the formation of structures known as molten globules. This modification induces an intermediate degree of denaturation, causing the proteins to deviate from their native state without undergoing a complete denaturation (Zhu et al., 2021). Table 4 presents the impact of pH-shifting under various conditions on a selection of plant proteins, and their subsequent functional and physicochemical properties. The resulting protein characteristics were greatly affected by the protein source but also whether the pH was shifted to acidic or basic conditions prior returning to neutral

Several studies have demonstrated significant pH-driven alterations in the secondary structures (α-helix, β-sheet, β-turn, and random coil) of rapeseed proteins (Li et al., 2020b), peanut (Li et al., 2020a), and soy proteins (Wang et al., 2023a). The extent of the changes depended on the protein type and pH conditions. Most treatments resulted in increased ratios of α-helix and random coil structures, while the β-turn structures were mostly decreased or remained unchanged. Furthermore, significant changes were observed in β-sheet structures, but there was no clear trend as to whether these increased or decreased. However, these alterations were not substantial, as the changes fell within the range of 0.1-5 % points, with particularly low changes in soy samples (≤0.4% points). Despite the minor alterations in secondary structures, notable changes by pH shift were observed in both the surface hydrophobicity and particle size of the proteins. This indicated that the hydrophobic regions of the proteins were either exposed or concealed, leading to fluctuations in particles size, depending on the pH conditions of the treatment.

Table 4. The effect of pH-shifting on functional properties of various plant protein isolates at their final pH conditions. Some of the values have been derived from graphical data and, as such, may not be precise.

Raw material	pH shift to	pH final	Solubility %	EAI / ES m^2g^{-1} / %	FC / FS % / %	WHC g/g	-SH µmol/g	PS nm	ZP mV	S_0
Pea [a]	Untreated	7	20	na / 96	230 / 83	4.9	1.3	na	na	na
	2	7	40	na / 94	205 / 70	1.7	1.0			
	10	7	24	na / 90	295 / 77	3.0	0.8			
	2 (80 °C)	7	48	na / 95	290 / 81	1.7	1.3			
	10 (80 °C)	7	52	na / 96	330 / 81	1.7	1.2			
Hemp [a]	Untreated	7	8	na / 24	210 / 76	2.1	1.2	na	na	na
	2	7	20	na / 54	210 / 67	3.0	2.2			
	10	7	20	na / 70	210 / 76	3.4	2.2			
	2 (80 °C)	7	18	na / 50	210 / 74	2.6	2.2			
	10 (80 °C)	7	14	na / 34	240 / 78	3.2	2.0			
Oat [a]	Untreated	7	5	na / 30	310 / 86	2.6	1.1	na	na	na
	2	7	10	na / 26	290 / 78	3.7	1.8			
	10	7	10	na / 30	310 / 84	4.8	1.8			
	2 (80 °C)	7	10	na / 24	220 / 75	3.4	1.8			
	10 (80 °C)	7	26	na / 94	295 / 81	5.8	1.8			
Rice [a]	Untreated	7	2	na / 18	287 / 92	2.9	1.2	na	na	na
	2	7	1	na / 18	240 / 88	2.8	1.2			
	10	7	3	na / 22	410 / 88	3.2	1.2			
	2 (80 °C)	7	2	na / 20	287 / 89	2.8	1.3			
	10 (80 °C)	7	4	na / 26	330 / 90	3.2	1.2			
Soy [b]	1	7	na	52 / 65	238 / 175	na	na	247	6.4	17000
	2	7		53 / 57	225 / 155			269	8.7	25000
	3	7		44 / 53	238 / 190			219	9.2	32500
	7 Control	7		35 / 44	112 / 90			192	-13	2000
	10	7		70 / 76	190 / 138			281	-11	2000
	11	7		65 / 57	220 / 145			272	-11	1000
	12	7		65 / 87	225 /155			251	-9.1	500
Rice [c]	7 Control	7	~0	na	na	na	na	na	-33	100
	12	7	~10						-28	125
	12 (90 °C)	7	~70						-33	260
Wheat [d]	Control 80 °C	na	20	8 / 37	na	na	na	na	-8.8	370
	12 (40 °C)	7	30	28 / 48					-7.2	1370
	12 (60 °C)	7	45	30 / 50					-9.0	1700
	12 (80 °C)	7	70	31 / 53					-7.2	2100
Rape-seed [e]	7 Control	7	9	na	na	na	na	325	-18	2050
	1.5	7	16					275	-11	2000
	2.5	7	19					310	-12	1900
	11.5	7	28					240	-21	1300
	12.5	7	32					230	-15	1800
Peanut [f]	7 Control	7	79	na	na	na	na	400	-32	na
	2	7	33					2000	-29	
	4	7	82					600	-33	
	10	7	90					200	-33	
	12	7	61					1000	-34	

Abbreviations: EAI = emulsion activity index, ES = Emulsion stability, FC = foaming capacity, FS foaming stability, WHC = water-holding capacity, OHC = oil-holding capacity, PS = particle size, ZP = zeta potential, S_0 = surface hydrophobicity, -SH = free thiol groups, na = not available, Control = treatment with identical conditions to pH-shifting samples excluding the change in pH conditions

References: [a] Tang et al. (2023), [b] Wang et al. (2023b), [c] Igartúa et al. (2024), [d] Xiong et al. (2023), [e] Li et al. (2020b), [f] Li et al. (2020a)

Numerous studies have investigated the combined effect of pH-shifting with heating at various temperatures (Igartúa et al., 2024; Tang et al., 2023; Xiong et al., 2023). For certain proteins, such as those from rapeseed and peanuts, solubility was most effectively increased when the pH was shifted towards basic conditions of pH 10. Conversely, proteins from pea benefit from acidic conditions of pH 2 (Li et al., 2020a; Li et al., 2020b; Tang et al., 2023). On the other hand, oat and hemp proteins showed improved solubility after both treatments, although the final solubility remained relatively low, ranging from 10-20% (Tang et al., 2023). Rice protein was an exception, as its solubility remained near zero despite attempts of combined heating at 80 °C and pH-shifting to 2 or 10 (Tang et al., 2023). However, when the pH was increased to 12 and the temperature to 90 °C, rice protein exhibited a drastic increase in solubility, from 0% to 70% (Igartúa et al., 2024). In

contrast, the solubility of hemp protein decreased upon heating. For peanut protein, a shift from pH 10 to 12 without heating begun to reduce the solubility. The decreased solubility of peanut protein under extreme pH conditions was associated with protein aggregation, which was caused by the exposure of hydrophobic groups and a decrease in free thiol groups (Li et al., 2020a). For wheat protein, a stepwise increase in temperature (40, 60, 80 °C) at pH 12 led to notable increase in solubility from 5% to 70%, which corresponded to improved emulsification properties (Xiong et al., 2023). In this scenario, there was a simultaneous increase in hydrophobicity and free thiol groups. This suggested that the protein structure was becoming more relaxed, rather than aggregating, indicating a successful transition to molten globule state.

While solubility improvements were observed, not all functional properties consistently improved. For instance, when the solubility of pea protein increased from 20% to 52%, the emulsion stability remained unchanged. However, the water and oil-holding capacities decreased, while the foaming capacity showed significant increase from 230% to 330%, with little change in stability (Tang et al., 2023). In contrast, oat protein clearly benefited from the increase in solubility from 5% to 26%. This was evident in the water-holding and emulsion stability, which increased from 2.6 to 5.8 g/g and 30% to 94%, respectively. Yet, little change was observed in the foaming properties. Interestingly, although the solubility of rice protein remained unchanged at pH 10 with combined heating at 80 °C, the foaming capacity underwent a substantial increase by 43% points.

In conclusion, pH-shifting significantly alters the functional and physicochemical properties of various plant proteins. However, its effectiveness varies according to protein source, pH conditions, and combined heating. While it mostly increases solubility, particularly at alkaline conditions combined with heating, its impact on functional properties is inconsistent. Therefore, a careful selection of pH-shifting parameters tailored to the specific applications of each protein source is essential for achieving a successful enhancement in functionality. While the effects of pH-shifting on solubility-dependent functional properties have been relatively well-studied, there is a noticeable lack of similar research concerning the formation of fibrous structure in meat analogues.

2.2.2 Enzymatic modification

Enzymes are widely used across diverse sectors of the food industry in various applications including dairy, bakery, juice, wine, brewing, meat, and oil processing, among others. Their application aims to improve, for instance, the techno-functional properties and stability of food products (Panyam and Kilara, 1996). Enzymes are also used in extraction processes to enhance both product yield and process efficiency (Hernández-Álvarez et al., 2023). The key advantages of employing enzymes for catalysing specific reactions lie in their high efficiency, specificity, and safety (Pang et al., 2021). The most common protein-specific enzymes for improving plant protein performance in food applications are hydrolysing exo- and endoproteases, crosslinking enzymes, and deamidating enzymes (Zeeb et al., 2017).

Limited enzymatic hydrolysis

Enzymatic hydrolysis reduces the molecular weight of the proteins by cutting the peptide bond between two adjacent amino acids. There are different types of hydrolysing enzymes (proteases) that may cut the peptide bonds from the end (exoprotease) or from the middle

(endoprotease) of the amino acid chain resulting in either free amino acids or peptides with various lengths, respectively (Gouseti et al., 2023; Wouters et al., 2016). This overview primarily focuses on limited enzymatic hydrolysis in relation to protein functionality, rather than enzymatic protein extraction. Table 5 shows the effect of limited hydrolysis on functional properties of selected plant proteins, including rapeseed, wheat, rice, and oat.

In general, an increase in the degree of hydrolysis leads to an increase in protein solubility. However, when considering functional properties, the size of the protein or peptide becomes a significant factor (Wang et al., 2006). Peptides that have undergone extensive hydrolysis are too short to form such networks required for properties such as foaming, emulsification, and gelation as these properties depend on the ability of proteins to encase air, oil or water droplets.

Various studies, as shown in Table 5, on wheat and rice proteins have demonstrated an increased solubility as a function of elevating degree of hydrolysis (Kong et al., 2007; Paraman et al., 2007; Xu et al., 2016). However, the improvement in emulsification and foaming properties only occurs up to a certain point. For instance, commercial wheat gluten was hydrolysed to degrees of hydrolysis ranging from 0-15%, which corresponded to solubility values of 15-80%. Yet, increased emulsion activity and stability, as well as foam stability, were only observed up to a degree of hydrolysis of 5%. Beyond this point, these properties began to decrease. Similarly, rice endosperm protein was subjected to limited hydrolysis at degrees of 5.2% and 13.5%, which corresponded to solubility values of 19% and 26%. However, the highest emulsification activity and stability were only observed at a 5% degree of hydrolysis. Furthermore, a 2% degree of hydrolysis was identified as the optimal point for emulsification stability of rice glutelin, which also corresponded to the highest surface hydrophobicity. Xu et al. (2016) associated the increased functionality with a more flexible protein structure. This flexibility exposed the inner hydrophobic regions due to an increase in random coil, α-helix, and β-sheet structures, and a decrease in β-turns. This structural transformation facilitated the absorption of protein to oil-water interfaces in emulsion formation. Regarding oat proteins, degrees of hydrolysis varied between 2-16%, leading to the highest emulsification activity at an 8% degree, while the stability was the highest at a slightly lower degree of hydrolysis of 6% (Zheng et al., 2020). At this extent of hydrolysis, the electrostatic repulsion (zeta potential) was the highest. For rapeseed protein ingredients, a range of 0 to 7.7% degree of hydrolysis was explored, and 3.1% degree resulted in the highest emulsification activity, stability, and foaming capacity and stability as well as water- and oil-holding capacities (Vioque et al., 2000).

Nieto-Nieto et al., (2014) observed an increase in gel hardness of oat protein ingredient through limited enzymatic hydrolysis using trypsin (serine protease) and flavourzyme (a blend of exo- and endoproteases). This was particularly evident when the gels were induced under alkaline conditions (pH 9). In summary, they studied four different enzymes that produced varying degrees of hydrolysis (5.3-7.1%) and resulted in peptides of different sizes. Interestingly, different enzyme had varying impacts on the gelation properties of the resulting hydrolysates, although the degrees of hydrolysis were similar. For instance, oat proteins treated with pepsin were unable to form a gel structure with a 5.5% degree of hydrolysis. In contrast, flavourzyme treated oat proteins, despite having a higher degree of hydrolysis at 7.1%, successfully formed robust gel structures. This phenomenon was attributed to the fact that pepsin, despite its lower degree of hydrolysis, resulted in peptides

of lower molecular weight compared to flavourzyme. This observation underscores the importance of the chosen enzyme type.

In conclusion, it can be inferred that partial hydrolysis improves the functionality of several plant proteins by increasing their flexibility through size reduction and exposed hydrophobic regions. However, an excessively high degree of hydrolysis can result in loss of functionality. Therefore, the optimal degree of hydrolysis is determined by the choice of enzyme and its ability to cleave proteins optimally, while avoiding over- or underdigestion.

Table 5. Effect of partial hydrolysis on functional and physicochemical properties of various plant protein ingredients. Some of the values have been derived from graphical data and, as such, may not be precise.

Raw material	Enzyme and treatment pH	DH %	pH	Solubility %	EAI / ES	FC/FS % / %	Other properties
							WHC/OHC
RPI [a]	Untreated	0	5.0*	na	1 / 1	18 / 0	1.3 / 0.6
RPI hydrolysates, filtrate	Alcalase at pH 8 (non-specific endo-protease)	3.1 5.0 7.1 7.7	8.0 8.0 8.0 8.0	na	50 / 75 39 / 28 29 / 19 25 / 1 (% / %)	69 / 68 58 / 61 59 / 52 59 / 45 at 20 min	5.9 / 1.6 5.1 / 1.3 5.4 / 1.3 5.5 / 1.4 (g/g)
WG [b]	Untreated	0	na*	15	0.35 / 0.18	na / 30	na
WG hydrolysates, soluble part	Alcalase at pH 8.5 (non-specific endo-protease)	5 10 15	7.0 7.0 7.0	65 70 80	0.65 / 0.39 0.5 / 0.19 0.3 / 0.1 (Abs 500 nm)	na / 70 na / 50 na / 45 at 20 min	na
REPI [c]	Untreated	0	7.0	12	0.31 / 15	na	na
REPI hydrolysates, whole material	Alcalase at pH 9 (non-specific endo-protease)	5.2 13.5	7.0 7.0	19 26	0.43 / 2 0.40 / 20.2 (m2/g / min)	na	na
							S_0
RG [d]	Untreated	0	7.0*	5	ES at 2% DH most resistant		240
RG hydrolysates, soluble part	Trypsin pH 8 (serine protease)	0.5 2 6	7.0 7.0 7.0	78 82 85	to droplet aggregation (7 days)		375 425 300
							Zeta potential
OPI [e]	Untreated	0	4.5*	na	na / na	na	na
OPI hydrolysates, soluble part	Alcalase at pH 8.5 (non-specific endo-protease)	2 4 6 8 12 16	7.0 7.0 7.0 7.0 7.0 7.0	na	20 / 2 23 / 2 22 / 15 27 / 4 19 / 10 27 / 2 (m2/g / h)	na	-2.9 -2.8 -7.8 -1.7 -1,0 -2.7 (mV)
							Gel hardness
OPI [f]	Untreated	0	9.0	na	na	na	0
OPI hydrolysates, whole material	Flavourzyme at pH 7 Alcalase at pH 8 Pepsin at pH 2 Trypsin at pH 8	7.1 5.8 5.5 5.3	9.0 9.0 9.0 9.0	na	na	na	5 na na 9 (N)

* Denotes the untreated raw materials, which are not comparable to their hydrolysates due to differences in pH or because the hydrolysates include only the soluble part of the treated raw material.

Abbreviations: RPI = rapeseed protein isolate, WG = wheat gluten, REPI = rice endosperm protein isolate, RG = rice glutelin, OPI = oat protein isolate, DH = degree of hydrolysis, EAI = emulsification activity index, ES = emulsion stability, FC = foam capacity, FS = foam stability, WHC = water-holding capacity, OHC = oil-holding capacity, , S0 = surface hydrophobicity, na = not available.

References: [a] Vioque et al. (2000), [b] Kong et al. (2007), [c] Paraman et al. (2007), [d] Xu et al. (2016), [e] Zheng et al. 2020, [f] Nieto-Nieto et al. (2014).

Enzymatic crosslinking

Enzymatic crosslinking catalyses the formation of covalent bonds between polypeptide chains or proteins. This process occurs either within the same molecule, forming intramolecular cross-links, or between two different molecules, forming intermolecular cross-links (Gouseti et al., 2023). There are several types of crosslinking enzymes including transglutaminase, tyrosinase, laccase and peroxidase. Transglutaminase is classified as a transferase enzyme while laccase, tyrosinase and peroxidase fall under the category of oxidoreductase enzymes (Gouseti et al., 2023; Isaschar-Ovdat and Fishman, 2018). Among these enzymes, transglutaminase is most commonly used in the food industry, making it the primary focus of this study. The reaction it catalyses involves the amino acid residues glutamine and lysine that react in the presence of water. Thes residues form a glutamyl-lysyl isopeptide bond between the γ-carboxamide and ε-amino groups (Gouseti et al., 2023). Table 6 provides a summary of the research conducted on the effects of transglutaminase on the functional properties of pea, oat, and faba bean proteins.

A common observation across studies on transglutaminase-treated protein isolates from pea, oat, and faba is that an increase in crosslinking generally led to a decrease in solubility at pH 7. (Nivala et al., 2017; Shen et al., 2022; Siu et al., 2002). Siu et al. (2002) reported a shift in the isoelectric point of oat protein from acidic to neutral conditions after transglutaminase treatment. This suggested that the increase in solubility of oat proteins at acidic conditions would provide an opportunity to use these polymerised proteins in beverage application. In contrast, no shift in isoelectric point was observed for pea protein (Shen et al., 2022). Both pea and oat proteins exhibited increased water- and oil-holding capacities when crosslinking was induced. Pea protein isolate also showed an increased emulsion activity and stability, whereas these properties decreased for oat proteins. On the other hand, the foaming and especially gelation properties of cross-linked oat proteins significantly increased, which was associated to increased random coil and reduced a-helix structures as well reduced surface hydrophobicity (Siu et al., 2002). Furthermore, also rapeseed proteins have demonstrated an increased heat-induced gelation after transglutaminase-mediated crosslinking (Pinterits and Arntfield, 2008).

In another study on oat proteins (Nivala et al., 2017), an increase in foaming capacity was also observed, but this was only after a certain degree of crosslinking had been achieved. Alongside the enhanced foaming capacity, they also noted an improvement in colloidal stability. Conversely, faba proteins exhibited decreased foaming properties with decreased solubility while the colloidal stability remained unchanged although the zeta potential values suggested an increased electrostatic stability. Both faba and oat proteins showed decreased particle size with an increasing degree of crosslinking.

In their further studies, Nivala et al. (2020) introduced a pre-heating treatment (90 °C, 30 min) prior to transglutaminase-mediated crosslinking of faba proteins, which notably improved its acid-induced gel strength as the degree of crosslinking increased. Following the heat treatment, faba proteins exhibited increased surface hydrophobicity and a shift in their secondary structure towards random coil. This alteration facilitated transglutaminase-mediated crosslinking as both hydrophilic and hydrophobic α and β-legumins participated in the reaction in contrast to the behaviour in unheated faba proteins. In addition, both emulsifying activity and stability improved, but the change was more apparent in the non-heated and cross-linked faba proteins.

In conclusion, the success of transglutaminase-mediated functionalisation is influenced by both degree of crosslinking and application of pre-treatments such as heating. Certain

functional properties, like colloidal stability, can be only improved for certain protein types. The improved in emulsification properties were associated with decreased hydrophobicity, robust gel strength in gelation required the exposure of hydrophobic groups. Therefore, also transglutaminase-mediated functionalisation requires careful selection of process parameters to be able to achieve an optimal degree of crosslinking for specific protein sources, thereby enhancing their functionality. Application of transglutaminase-mediated functionalisation to improve fibrous structure formation in meat analogues is introduced in section 2.3.

Table 6. Effect of transglutaminase treatment on functional and physicochemical properties of various plant protein isolates. Some of the values have been derived from graphical data and, as such, may not be precise.

Raw material	Dosage protein-wise	Solubility at pH 7 %	EAI / ES m^2g^{-1} / min	FC / FS % / %	WHC / OHC g/g	CS	Gelation Pa (G')	ZP mV	PS nm	S_0
PPI [a]	0 1.0 %	80 30	59 / 48 95 / 58 (%)	na	2.7 / 2.8 5.3 / 3.1	na	No change	na	na	202 73
OPI [b]	0 0.01 U / mg	40 5	21 / 6.9 7.5 / 4.3	64 / 55 140 / 32	1.0 / 1.6 1.5 / 4.5	na	340 5740	na	na	na
OPI [c]	0 10 100 1000 nkat / g	16 21 19 19	na	Ctrl* ↘ / - ↗ / - ↗ / -	na	Ctrl* - ↗ ↗	na	-24 -27 -29 -32	255 135 101 103	na
FPI [c]	0 10 100 1000 nkat / g	83 77 64 60	na	Ctrl* ↘ / - ↘ / - ↘ / -	na	Ctrl - - -	na	-30 -3 -36 -38	145 100 64 52	na
FPI [d]	0 1000 H-0 H-1000 nkat / g	na	25 / 18 31 / 35 27 / 26 29 / 23	na	na	na	10 nkat: 323 100 nkat: 3534	na	na	 181 162 504 435

* These properties are expressed as no change (-), increased (↗), or decreased (↘) values in comparison to the non-treated samples (ctrl)

Abbreviations: PPI = pea protein isolate, OPI = Oat protein isolate, FPI = faba protein isolate, TG = transglutaminase, Ctrl = control treatment, H = pre-heating at 90 °C, WHC = water-holding capacity, OHC = oil-holding capacity, ZP = zeta potential, PS = particle size, EAI = emulsifying activity index, ES = emulsion stability, FC = foam capacity, FS = foam stability, S_0 = protein surface hydrophobicity, na = not available.

References: [a] Shen et al. (2022), [b] Siu et al. (2002), [c] Nivala et al. (2017), [d] Nivala et al. (2020).

Enzymatic deamidation

Enzymatic deamidation is a biochemical process that converts the amide group in the side chain of asparagine or glutamine amino acid residues into carboxyl group resulting in aspartic acid and glutamic acid (Gouseti et al., 2023; Zhang et al., 2021a). Table 7 provides a summary of research on enzymatic deamidation conducted on selected plant protein sources including, including oats, wheat, rice, and soy.

The general trend from these studies shows that deamidation significantly enhances the solubility of proteins in oats, wheat, rice, and soy, increasing it from an initial range of 0-32% to much higher range of 70-90% (Jiang et al., 2015; Liu et al., 2011; Suppavorasatit et al., 2011; Yong et al., 2006). However, the solubility of pea proteins only increased to a certain extent (Fang et al., 2020) or even decreased (Shen et al., 2022) when compared to the unmodified pea protein. It is worth mentioning that the unmodified pea protein in

Shen et al. (2022) already exhibited notably high solubility of 80% that decreased after deamidation to 60%, which in turn resulted in decreased gelation and oil-holding properties accompanied with reduced surface hydrophobicity and increased free thiol groups.

Regardless of the differences in protein solubility trends of pea proteins, the emulsifying properties of oat, wheat, pea, and soy proteins all improved after deamidation. For instance, deamidation at degree of 59% increased emulsion stability of oat proteins from only 2 hours to 30 days. Research on the effects of deamidation on foaming and gelation properties on plant proteins is scarce. However, one study by Suppavorasatit et al. (2011) reported an increase in foaming capacity from 26% to 55% at deamidation degree of 33%. Furthermore, deamidation of, wheat, rice, pea, and soy protein isolates resulted in an increased colloidal stability, observed as an increased turbidity accompanied with reduction in sediment (Fang et al., 2020; Liu et al., 2011; Suppavorasatit et al., 2011; Yong et al., 2006). The impact of deamidation on the physicochemical characteristics of these proteins has been less explored. However, in the case of rice and pea proteins, an increase in electrostatic repulsion (measured as zeta potential) from -15 mV and -24mV up to -29 mV and -35 mV was observed. Additionally, Fang et al. (2020) and Jiang et al. (2022) reported increased surface hydrophobicity for pea and soy proteins after deamidation along with the afore mentioned improved functionality.

Table 7. Effect of enzymatic deamidation on functional and physicochemical properties of various plant proteins. Some of the values have been derived from graphical data and, as such, may not be precise.

Raw material	DD %	Solubility %	EAI / ES	ZP mV	S_0	Description
OPC [a]	0 15 42 59	32 30 40 90	na / 2 hours na / day na / 7 days na / 30 days	na	na	Deamidation decreased intra- and intermolecular β-sheets (22.1 → 12.7% / 2.8 → 1.4%) as well as β-turns (20.1 → 15.4%), increased α-helices (28.6 → 40.0%) and random coil (25.5 → 30.4%) structures
WG [b]	0 72	0 70	na / 0 hours na / 8 days	na	na	Deamidation increased α-helix, β-turn, and random coil structure while it decreased β-sheets → increased colloidal stability and emulsion stability
RG [c]	0 26 52	8.6 56 76	na / na	-15.9 -19.2 -29.2	na	Deamidation decreased random coil and α-helix structures, increased β-turns while β-sheets remained almost the same → reduction of macromolecular complexes → increased colloidal stability
PPI [d]	0 56 60	15 65 45	na / na	-24.1 -32.4 -35.0	2094 2729 2795	Deamidation decreased β-sheets, increased β-turns and α-helices → protein unfolding → exposed hydrophobic groups → increased colloidal stability
PPI [e]	0 PG 1%	80 60	59 / 48 63 / 52 (% / %)	na	202 162	Deamidation decreased β-sheet and random coil structures, and increased β-turns and α-helices, → increased free -SH → increased WHC, decreased OHC
PPI [f]	0 33 44 52	25 65 68 70	20 / 18 27 / 45 31 / 40 29 / 41 (m^2g^{-1} / min)	na	na	Deamidation increased β-sheets and random coils, decreased α-helices and β-turns remained same. It increased degree of hydrolysis (0% to 11%) → increased colloidal stability → increased foaming capacity (26% to 55%)
SPI [g]	0 42	na	45 / 92 80 / 95 (m^2g^{-1} / %)	na	100 490	Deamidation increased degree of hydrolysis, particle size, and high-molecular-weight macromolecules, while it decreased free -SH groups.

Abbreviations: OPC = oat protein concentrate, WG = wheat gluten, RG = rice glutelin, PPI = pea protein isolate, SPI = soy protein isolate, PG = protein-glutaminase, DD = deamidation degree, EAI = emulsion activity index, ES = emulsion stability, ZP = zeta potential, S0 = surface hydrophobicity, WHC = water-holding capacity, OHC = oil-holding capacity, SH = free thiol groups, na = not available.

References: [a] Z. Jiang et al. (2015), [b] Yong et al. (2006), [c] Liu et al. (2011), [d] Fang et al. (2020), [e] Shen et al. (2022), [f] Suppavorasatit et al. (2011), [g] Jiang et al. (2022).

Regarding secondary structures (Table 7), the general trend observed was a decrease in the number of β-sheets after deamidation for oat, pea, and wheat proteins, while for soy proteins, it actually increased. Conversely, α-helices and β-turns generally increased after deamidation, with a few exceptions specific to protein source. For instance, β-turns in oat

proteins and α-helices in rice and soy protein did not follow this trend. As for the random coil structures, no consistent trend was observed across the studied protein sources.
The deamidation mechanism appears to vary depending on the protein source. For instance, Liu et al. (2011) observed a significant reduction in high-molecular weight aggregates after deamidation in the case of rice glutelin. They suggested that some of the intermolecular disulphide bridges, which hold together the rice glutelin subunits, were broken. They suggested that this breakdown accompanied with the changes observed in secondary structures could lead to a more flexible protein form that facilitated the solubilisation and subsequent improvement in functionality. On the other hand, Jiang et al. (2022) reported an increase in both particle size and number of high-molecular weight macromolecules (> 400 kDa) when they studied deamidated soy proteins. They suggested that the increase in exposed hydrophobic regions led to protein aggregation in deamidated soy proteins. This aggregation was then stabilised through the formation of disulphide bonds, as evidenced by the observed reduction in the amount of free thiol groups.

In summary, the deamidation process can lead to improved functional properties of different plant sources through improved solubility, which may be achieved through varying changes in the molecular structure and behaviour of these proteins. In some rare cases, it may also reduce the functionality through decreased solubility.

2.2.3 Fermentation

One key approach to improve plant protein functionality involves fermentation. This method has been explored across a broad spectrum of food applications, including baking, deflavouring, sausage-analogues, meat analogues, pudding, and dairy alternatives (Table 8).

In the scope of baking, Galle et al. (2010) proposed the use of *Weissella confusa*, a bacterium capable of converting sucrose into exopolysaccharides like dextran, to improve the quality of both conventional and gluten-free breads. This proposed mechanism was further studied by Galli et al. (2020), who demonstrated an increase in the viscosity of chickpea sourdough fermented with *W. confusa*. Wang et al. (2022) extended this concept by using *W. confusa* to produce dextran during fermentation of rapeseed proteins. This fermented rapeseed protein was then incorporated into wheat flour dough to create high-protein breads, resulting in improved textural properties such as increased bread volume and reduced crumb hardness.

Xing et al. (2020) demonstrated the benefits of spontaneous solid-state fermentation on a protein-enriched chickpea ingredient. This process led to improved functional properties and nutritional value, including an increased water-holding capacity and a reduction in phytic acid content. In the context of deflavouring, Shi et al. (2021) successfully used lactic acid bacteria-mediated fermentation to eliminate the unpleasant aroma in pea protein isolates. Interestingly, they observed that the oil-holding capacity and foaming properties improved after certain periods of fermentation, while at other times, the opposite effect was observed. Valtonen et al. (2023) reported similar findings, demonstrating a reduction in the pea-like odour in plant-based sausages produced through a combination of fermentation and extrusion.

Kaleda et al. (2020) used fermentation in an attempt to improve the sensory and nutritional properties of dry extrudates from a pea and oat protein blend. However, they observed a reduction in processability and a lower tendency to form a fibrous structure. Pöri et al. (2023) successfully reduced the high antinutrient content in sunflower-derived protein concentrate through fermentation with *Lactobacillus helveticus*, aiming to combine it with pea protein for extrusion processing. Additionally, fermentation also reduced the fibrous structure formation and induced a sour taste, not suitable for meat analogue applications. However, they found that adjusting the pH to 7 reduced the sour taste and simultaneously improved structure formation. Despite these findings, systematic research on fermentation as functionalisation strategy to improve meat analogue properties is limited.

Table 8. Effect of fermentation on plant protein ingredients in various food applications.

Raw material	Strain	Initial pH	Final pH	Application	Description
Chickpea flour [a]	*Weissella confusa*	6.20-6.34	4.38-4.65	Gluten-free sourdough	Increased levels of final viscosity and EPS (dextran).
Chickpea protein concentrate (dry fractionated) [b]	*Pediococcus* spp. (solid-state fermentation)	6.6	4.2	Clean-label sourdough	Increased protein hydrolysis, decreased foam capacity, increased WHC.
Rapeseed protein isolate or concentrate [g]	*Weissella confusa*	6.1 or 6.2	5.6 or 5.5	High-protein bread	Decreased number of condensed tannins in RPI. Increased number of condensed tannins in RPC. Increased viscosity. Increased dextran levels. Increased degree of hydrolysis (RPI) while decreased DH (RPC). Breads with improved textural properties
Oat protein concentrate (dry fractionated) [c]	*Lactobacillus delbrueckii* subsp. *Bulgaricus* and *Streptococcus thermophilus*	7.0	4.2	Yoghurt analogue	Strengthened rheological properties
Pea protein concentrate [e]	*Lactobacillus plantarum*	na	na	Ingredient	Increased number of tannins and phenols. Reduction of sulphur containing amino acids. Increased protein digestibility.
Pea protein isolate [f]	*Lactobacillus plantarum*	7.56	6.59	Ingredient	Improved sensory properties (decreased beany aroma and flavour as well as decreased bitterness). Decreased water-soluble protein, EHC. Whether the functional properties (OHC, and foaming properties) increased or decreased depended greatly on fermentation time.
Okara (soy presscake) [d]	*Lactobacillus plantarum* or *Lactobacillus acidophilus*	4.9	4.57 or 4.14	Meat patty analogues	Increased WHC and OHC. Reduction of hardness and increased juiciness of meat analogues.
Pea protein isolate and concentrate [h]	*Lactiplantibacillus plantarum*	6.4	4.3	Sausage analogue	Increased sour, umami, and sweet taste. Decreased pea-odour. Increased chewiness.
Pea and oat protein blend [i]	Commercial mixture of lactic acid bacteria			Meat analogue	Fermentation caused lower tendency to form fibrous structures. Increased water-solubility index of dry-extrudate.
Pea protein isolate and sunflower protein concentrate blend [j]	*Lactobacillus helveticus*	6	4.5	Meat analogue	Fibrous structure formation lost after fermentation but regained when shifted to pH 7. Highest bitterness and lowest fibrousness in fermented sample

Abbreviations: na = not available

References: [a] Galli et al. (2020), [b] Xing et al. (2020), [c] Brückner-Gühmann et al. (2019), [d] Razavizadeh et al. (2021), [e] Çabuk et al. (2018), [f] Shi et al. (2021), [g] Wang et al. (2022), [h] Valtonen et al. (2023), [i] Kaleda et al. (2020), [j] Pöri et al. (2023).

2.3 Meat analogues

Meat analogues are food products that aim to mimic the muscle fibres of animal meat, and they can be crafted using technologies including high-moisture extrusion processing (HMEP), shear cell technology, and dry extrusion processing (Dekkers et al., 2018). This survey primarily concentrates on meat analogues produced using HMEP with relevant examples regarding shear cell technology, two well-established methods that yield products with a high moisture content, typically between 45% and 70% (Table 9). The focus is on high-moisture meat analogues as they closely mirror the water content of cooked animal meat, which usually ranges between 56-65% (Anonymous, 2024). In contrast, dry extrusion produces sponge-like structures that require rehydration before use, making them less ideal for mimicking the texture and juiciness of animal meat. One of the major challenges in producing plant-based meat analogues is to achieve good fibrous structure and desirable sensory attributes, that match those of the animal meat. Therefore, this study excludes the dry meat analogue products that require hydration.

Both HMEP and shear cell technologies are thermomechanical processes capable of producing similar structures, but they employ different type of equipment (Cornet et al., 2022). A key difference between them is that HMEP operates as continuous process, while shear cell technology functions in a batch-wise manner. HMEP involves a temperature-adjustable barrel equipped with screws that facilitate the movement and shearing towards a die. This process requires a long slit cooling die, to be able to produce temperature gradients that elongate the protein melt. In contrast, the shear cell technology is also able to produce elongated fibres, but within a rotating cone-in-plate or cylindrical system. It follows the same three operations as HMEP involving mixing, thermomechanical shearing, and subsequent cooling. However, this process is not yet commercially available, which is why the focus of this work is on HMEP.

Table 9 highlights the extensive use of soy, pea, and wheat protein ingredients in the development of high-moisture meat analogues, while research on protein ingredients from rapeseed, oats and rice is notably limited. Furthermore, the majority of the raw materials in Table 9 are highly concentrated proteins, with contents exceeding 70%. There are only few exceptions where protein content falls below this value, which highlights the current situation in research that focus mainly on protein isolates in the creation of meat analogues although using protein concentrates would have both nutritional and environmental benefits. Furthermore, a predominant focus of the studies lies in the optimisation of the processing parameters or the simulation of the extrusion process for predictive purposes. Additionally, numerous studies attempted to explain the protein-protein interactions leading to fibrous structure formation during extrusion processing. However, only a limited number of studies have aimed to enhance the fibrous structure formation of protein concentrates, which are often incapable of forming fibres when used as a sole ingredient.

Table 9. Recent studies on plant-based meat analogues produced by high-moisture extrusion processing and shear cell technology.

HIGH-MOISTURE EXTRUSION PROCESSING									
Raw material	**Protein dm %**	**Screw diam. mm**	**L/D ratio**	**Screw speed rpm**	**Water content %**	**Temperature profile in barrel from feeding to die section °C**	**Cooling die T °C**	**Description**	**Main findings of the study**
Soy protein concentrate [a]	67	25.5	29:1	180-800	57-63	40/60/100/100/100/100-160	20	Modelling polymerisation in closed cavity rheometer	Structural changes insoluble and soluble polysaccharides have major impact on rheological properties and subsequent formation of fibrous structures
Soy protein isolate [b]	90	36	40:1	280	55	35/60/80/110/140/140/140/140/110	70	Insoluble dietary fibre addition	Partial substitution of protein with insoluble dietary fibre → improved fibrous structure until 20% inclusion rate
Soy protein isolate + oat b-glucan [c]	95	11	40:1	150	45	70/90/110/70-130/70/50	No	Glycosylation	Oat b-glucan conjugates were produced using HMEP without cooling die
Soy protein isolate + whey protein concentrate [d]	90, 80	11	40:1	600	57	25/50/90/110/133-155/ 138-159/138-160	-10	Blending ingredients	Anisotropic structures soy protein isolate increased with whey protein addition
Soy protein isolate + wheat gluten (70:30) [e]	91, 85	25	24:1	350	57	30/60/90/130/130/130	30	Polysaccharide addition	Polysaccharide inclusion enhanced thermal stability, and fibrous structures
Soy protein concentrate + wheat gluten [f]	70, 75	25.0	28:1	400	57	20/50/80/11/150/170/150	25	Blending ingredients	Blending of soy and wheat proteins increased disulphide bonding and texturisation degree → improved fibrousness
Soy protein isolate + hemp protein concentrate [g]	97, 77	20	40:1	300-800	65-74	40/60/80/100	nd	Maximum substitution of soy by hemp	Substitution of soy protein isolate by hemp protein concentrate gradually decreased fibrous structure formation
Wheat gluten [h]	83	25.5	29:1	300	40	40/60/80-100/90-100/90/100/120/ 90-170	0-80	Process conditions, die type	Variations in barrel temperatures were responsible for changes in polymerisation rather than die length and screw config
Wheat gluten [i]	83	25.5	29:1	180-800	54	40/60/90/100/100/100-155	50	Effect of process parameters	Polymerisation and anisotropic structure formation increased with elevated temperatures, but also other parameters affected
Pea protein isolate + rapeseed protein concentrate (50:50) [j]	88, 57	20	40:1	500-900	60-70	40/80/130/150	nd	Blending and process conditions	Higer moisture content had higher effect on structure than screw speed → both decreased chewiness and hardness
Pea protein isolate + destarched oat protein concentrate, wheat gluten [k]	83, 37	11	40:1	300	nd	50/60/90/120/130/140-170/ 140-170/115	30	Inclusion of destarched oat, CCR simulation	Successful inclusion of de-starched oat, temperature sweep data in CCR correlated with structure formation T in HMEP
Pea protein isolate (3 x different commercial) [l]	85, 87, 83	16	25:1	150	55	40/60/80/100/100-160	80	Relation of protein properties to extrusion responses	Regardless of different protein characteristics, viscosity behaviour and energy input → all ingredients showed similar well-aligned fibrous structures
Pea protein isolate [m]	82	nd	40:1	100	65, 70	90/100/110/120/130	45	Simulation of protein behaviour in barrel	With increased temperatures pea proteins transitioned from spherical form to fibrous structure, lost their original rigid secondary and tertiary structures, but formed new higher energy cross-links in HMEP

Raw material	Protein dm %	Screw diam. mm	L/D ratio	Screw speed rpm	Water content %	Temperature profile in barrel from feeding to die section °C	Cooling die T °C	Description	Main findings of the study
Pea protein isolate [n]	76	36	24:1	162	54	40/120/169/145/140	50	Cysteine inclusion	L-cysteine addition increased fibrous degree up until 0.09% inclusion rate after which it started to decrease fibrousness
Pea protein isolate (commercial) [o]	84	20.0	40:1	800	67	40/80/130/150	20	Exploring new plant-based raw materials for HMEP to replace soy	All ingredients produced fibrous well-layered structures in HMEP, and they all required different processing condition for successful structuring. Texture properties were influenced by ash, fibre, fat, protein content, and water-holding capacity properties of the protein isolates and concentrates.
Pea protein isolate (experimental) [o]	83	20.0	40:1	400, 600	67	40/80/130/150	20		
Faba protein concentrate (commercial) [o]	62	20.0	40:1	400-600	60-62	40/60/130/150	20		
Faba protein isolate (experimental) [o]	90	20.0	40:1	800	62	40/60/110/130	20		
Faba protein isolate + faba protein concentrate [p]	80, 55	24	28:1	400	60-65	25/40/80/100/120/150/150	40-80	Maximum inclusion of faba concentrate	Higer inclusion level of faba concentrate reduced hardness, chewiness, gumminess, cutting strength, but increased lightness and water absorption capacity
Faba bean protein concentrate [q]	64	20	40:1	900	nd	120-140 (last zone)	20	Structuring dry fractionated faba ingredient	Successful production of meat analogues with good bite-feeling solely from faba concentrate with optimum T at 130-140 °C
Lupin protein isolate + lupin protein concentrate + iota carrageenan (68:29:3) [r]	82, 55	27.0	37:1	800	55	40/60/90/120/160/160/160/140/120	30	Control extrusion	Spirulina flour could be added up to 50% into lupin-based meat analogue. Extrusion parameters affected structure properties, and in vitro protein digestibility. Spirulina addition yielded in dark green colour.
Lupin protein isolate + lupin protein concentrate + spir-ulina + iota carrageenan [r]	82, 55, 72	27.0	37:1	500-1200	50, 55, 60	145/160/175 (zones 5-7 from total of 9 zones)	30	Spirulina addition	
Lupin protein isolate + lupin protein concentrate (50:50) [s]	82, 55	26.7	37:1	400-1800	40-68	40/60/90/120/135-180 140/120	30	Extrusion parameters and responses	Successful production of lupin protein meat analogues. Water feed affected responses the most, followed by screw speed, with barrel T being least influential
Hemp protein concentrate + oat fibre residue [t]	51	20	40:1	500-900	60-66	40/70/110/120/130/130-150	60	Blending and process parameters	Mixture resulted in coarse looking, but fibrous extrudates. Higher temperatures and screw speeds were required for stronger fibres while increased water led to weaker structures
Peanut protein [u]	61	36	24:1	210	55	25/60/90/155/155/110	70	Added trans-glutaminase through feeder	Increased fibrous degree and highest tensile strength at 0.2% dosage (tested range 0-0.3%).
Soy protein isolate [v]	92	36	24:1	240	60	60/80/145/150/130	70	Trans-glutaminase	Fibrous degrees and appearances of the fibres varied according to protein source. Rice showed no fibres, wheat only prior to TG, while pea, peanut, and soy isolate showed increased fibrousness. Soy protein concentrate showed similar fibres.
Soy protein concentrate [v]	73	36	24:1	240	60	60/80/145/150/130	70		
Wheat gluten [v]	88	36	24:1	240	60	60/80/145/150/130	70		
Pea protein isolate [v]	85	36	24:1	240	60	60/80/145/150/130	70		
Rice protein isolate [v]	87	36	24:1	240	60	60/80/145/150/130	70		
Peanut protein [v]	55	36	24:1	240	60	60/80/145/150/130	70		

SHEAR CELL TECHNOLOGY									
Raw material	**Protein dm %**	**Screw diam. mm**	**L/D ratio**	**Rotation rate rpm**	**Water content %**	**Processing temperature and time during shearing °C**	**Cooling die T °C**	**Description**	**Main findings of the study**
Soy protein isolate + wheat gluten [w]	90, 81	na	na	5-50	69	90-110 for 5-25 min	90	Demonstration in Couette cell	Well-aligned fibrous structures were formed at 95-100 °C
Soy protein fractions + soy flour (70:30) [x]	68-75, 37	na	na	30	56	140 for 30 min	RT in 5 min	Pre-heating of soy protein	Soy protein fractions toasted at 150 °C resulted in the highest water-holding capacity with intermediate solubility, which led to most distinct fibrous structures
Soy protein concentrate [y]	74	na	na	30	59	95-150 for 15 min	25 in 15 min	Benchmarking rapeseed proteins against soy	Both rapeseed and soy proteins initiated fibrous structure formation at 140 °C, aligning better at 150 °C. Rapeseed fibres were thinner and more pronounced.
Rapeseed protein concentrate [y]	56	na	na	30	59	95-150 for 15 min	25 in 15 min		
Soy protein isolate + wheat gluten (50:50) [z]	86, 78	na	na	39	60	95-140 for 15 min	25 in 5 min	Comparing structuring of pea and soy with gluten	Pea-based blends showed fibrous structures at 120 °C and higher while soy blends yielded similar structures at wider T range. Optimal structures were comparable to chicken meat.
Pea protein isolate + wheat gluten (50:50) [z]	84, 78	na	na	39	60	95-140 for 15 min	25 in 5 min		

Abbreviations: HMEP = high-moisture extrusion processing, RT = room temperature T = temperature, CCR = closed cavity rheometer, dm = dry matter basis, L/D = length to diameter ratio, na = not applicable, nd = not disclosed. Protein contents that were not reported as dry matter basis in the original reference were calculated based on the given moisture content in the reference data.

References: [a] Pietsch et al. (2019a), [b] Deng et al. (2023), [c] Li and Li (2023), [d] Wittek et al. (2021), [e] Wang et al. (2023a), [f] Chiang et al. (2019), [g] Zahari et al. (2020), [h] Pietsch et al. (2017), [i] Pietsch et al. (2019b), [j] Zahari et al. (2021), [k] Immonen et al. (2021), [l] Osen et al. (2014), [m] Sun et al. (2022), [n] Peng et al. (2022), [o] Ferawati et al. (2021), [p] Kantanen et al. (2022), [q] Do Carmo et al. (2021), [r] Palanisamy et al. (2018), [s] Palanisamy et al. (2019), [t] Zahari et al. (2023), [u] Zhang et al. (2021b), [v] Zhang et al. (2023), [w] Krintiras et al. (2015), [x] Geerts et al. (2018), [y] Jia, Curubeto, et al. (2021), [z] Schreuders et al. (2019)

2.3.1 High-moisture extrusion processing

The HMEP is a technique specifically designed for the production of high-moisture meat analogues that exhibit fibrous structures (Figure 3). This method setup typically includes several components, including a feeder, a water pump, an extruder barrel with multiple adjustable heating zones, and extruder screws equipped with a diverse set of kneading and conveying elements (Cornet et al., 2022; Zhang et al., 2015). Additionally, a long slit cooling die is attached to a die body at the end of the barrel. The extrusion process itself can be divided into five main stages: feeding, mixing, melting, cooling and discharge, as outlined in the review by (Schmid et al., 2022).

During the feeding phase, plant-based raw materials are fed into the extruder barrel, where the temperature is maintained at a relatively low level between 25-60 °C to prevent the material from burning. In the subsequent mixing phase, the dry powder is fed to the screws from the feeder and is then mixed with water coming from an external pump. This is done under relatively low temperatures, typically less than 100 °C, to avoid exceeding the boiling point of water. Next, in the melting phase, the conveying screw elements transport the formed paste towards the end of the barrel, where the pressure starts to build up and reaches the highest point before entering the cooling die. The temperature profile of the barrel is usually set to gradually increase from the beginning to the end, where the die body is attached. Alternatively, the barrel temperature can be set to rise from a low to high temperature and then decline again at the end of the barrel to facilitate the transfer from barrel to the cooling die. Finally, during the cooling and discharge phases, the protein-rich paste enters and exits the long slit cooling die, where it is cooled and shaped into an anisotropic fibrous structure through a complex interplay of different physicochemical interactions and spinodal phase separation (Liu and Hsieh, 2007; Sandoval Murillo et al., 2019; Zink et al., 2024).

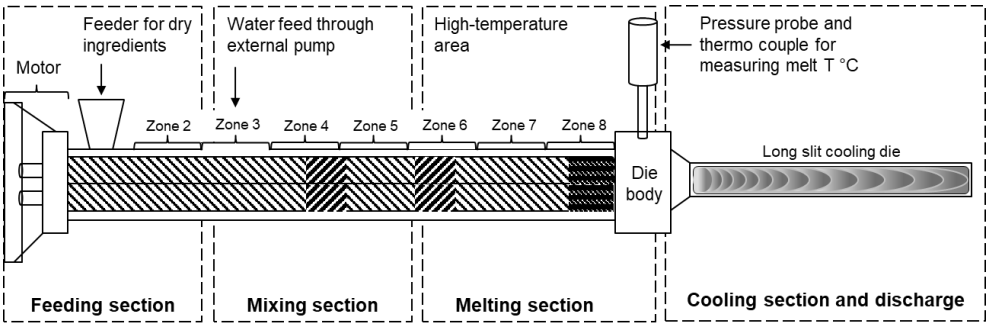


Figure 3. Equipment design and different extrusion phases during high-moisture extrusion processing for creation of fibrous high-moisture meat analogues.

2.3.2 Condition for fibrous structure formation

The formation of fibrous structures in protein concentrates and isolates is subject to specific operational conditions. The primary focus of this section is to enlighten the range of temperatures at which fibrous structures are induced for various plant proteins presented in Table 9. For instance, Pietsch et al. (2019a) found that soy protein concentrate forms fibrous structures at melt temperatures of 121 °C at a screw speed of 180 rpm, and at 111 °C with a higher screw speed of 500 rpm. These conditions corresponded to operational

temperatures of 140°C and 100°C, highlighting the importance of understanding that the operational barrel temperature does not equal to the protein melt. Therefore, comparisons between process temperatures across various studies require the information of the melt or product temperature. Similarly, Wittek et al. (2021) reported a melt temperature of 115 °C at 600 rpm, corresponding to operational temperature of 133 °C as the fibrous structure formation temperature for soy protein isolate. Wheat gluten, however, forms a fibrous structure at a higher melt temperature of 145°C (Pietsch et al., 2017). Pea protein isolate requires extrusion temperatures above 120°C to form a fibrous structure and continues towards stronger structures at elevated temperatures (Immonen et al., 2021; Osen et al., 2014). However, pea protein concentrate showed minimal ability to form a fibrous structure, even at higher extrusion temperatures of 160-140 °C at the barrel end (Nyyssölä et al., 2021).

Do Carmo et al. (2021) reported optimal conditions for fibrous structure formation in faba bean protein concentrate between 130-140°C (operation temperature at the barrel end). Interestingly, rapeseed protein concentrate with only 56%-dm protein content was able to form prominent fibrous structure at a shearing temperature of 150 °C using shear cell technology (Jia et al., 2021a). Moreover, some fibres were visible already at 140 °C. Lastly, a blend of lupin protein isolate, and concentrate started to form fibrous structures at melt temperature of 121 °C corresponding to operation value of 155 °C at 1200 rpm (Palanisamy et al., 2019).

As evidenced from the studies above, the fibrous structure formation in different raw materials vary in terms of temperature and protein content. When the protein concentration is low, the resulting structure is rather isotropic and exhibits weak fibres. To overcome this challenge various studies (Chiang et al., 2019; Krintiras et al., 2015; Schreuders et al., 2019; Wittek et al., 2021) demonstrated the benefits of blending different protein-rich raw materials to obtain more prominent fibrous structures. Blending soy protein isolate with whey protein concentrate improved the fibrous structure in a way that the fibre network became thinner and got more evenly distributed resulting in higher cutting strength and anisotropy index. This was accompanied with lower viscosity in the blended raw material.

2.3.3 Protein-protein interactions

The conversion of plant protein structure from a paste to fibrous meat analogue can be studied using dead-stop operation. Yao et al. (2004) illustrated this transformation with digital images of the macrostructures across different extruder zones, observing a gradual shift towards fibrous alignment. Their research showed that the formation of aligned fibres initiated in the final heating zone, corresponding to the melting section, just before reaching the cooling die where the fibres were further aligned and stabilised. Later research elaborated this at the molecular level, conducting solubilisation studies with different extraction solvents to investigate various protein-protein interactions (Liu and Hsieh, 2008). The studied solvents included combinations that break interactions such as covalent disulphide bonds along with non-covalent hydrogen bonds and hydrophobic interactions. These solvents were compared against a solvent system that broke all interactions, resulting in complete protein solubility throughout all extrusion sections. A sharp decrease in solubility occurred when the protein paste transitioned from the mixing section to the

melting section. This was linked to the formation of new disulphide bonds, as the extraction solvent being used disrupted all other interactions excluding disulphide bonds.

Building on previous research, Zhang et al. (2022b) extended the understanding of disulphide bond interactions during HMEP. They studied the formation of these bonds during various extrusion section using Raman spectroscopy, which can distinguish between the proportions of intra- and intermolecular disulphide bonds. In general, the proportion of intermolecular disulphide bonds was significantly lower than the intramolecular proportion across all extrusion section. Interestingly, the number of intermolecular disulphide bonds gradually increased from the mixing section to cooling section and extrudate. This increase corresponded to the formation of well aligned fibrous structure emphasising the importance of intermolecular interactions for crafting the fibrous structure of meat analogues.

Beyond disulfide bonds, numerous studies have reported other interactions contributing to the structure formation in HMEP such as non-covalent hydrogen bonding and hydrophobic interactions (Liu and Hsieh, 2008; Osen et al., 2015; Zhang et al., 2022b). However, the literature reveals a contradiction regarding the relative importance of different types of protein interaction during HMEP. Some studies emphasise the role of covalent disulphide bonds (Liu and Hsieh, 2008; Osen et al., 2015), while others suggest that non-covalent interactions are more important (Chen et al., 2011; Lin et al., 2000). The contradiction observed across these studies could potentially be attributed to the variation in the solvents utilised in solubility analytics regarding the protein interactions. Liu and Hsieh, (2008) convincingly demonstrated the importance of carefully designed solvent systems, including the isoelectric focus buffer that disrupts all interactions. They compared this system with more commonly used simpler buffers and were able to demonstrate the related limitations. Thus, their conclusion was that while non-covalent bonds are important in maintaining the secondary and tertiary structures, disulphide bonding is the key interaction in preserving the rigid structure of an extrudate and facilitating the formation of a fibrous structure. A similar conclusion has been reported for protein interactions using low-moisture extrusion (Hager, 1984). Regarding the formation or disruption of covalent peptide bonds, several studies have suggested that these interactions are resistant to changes during extrusion processing (Hager, 1984; Liu and Hsieh, 2008; Osen et al., 2015). Figure 4 presents a rough illustration of these interactions in a molecular level.

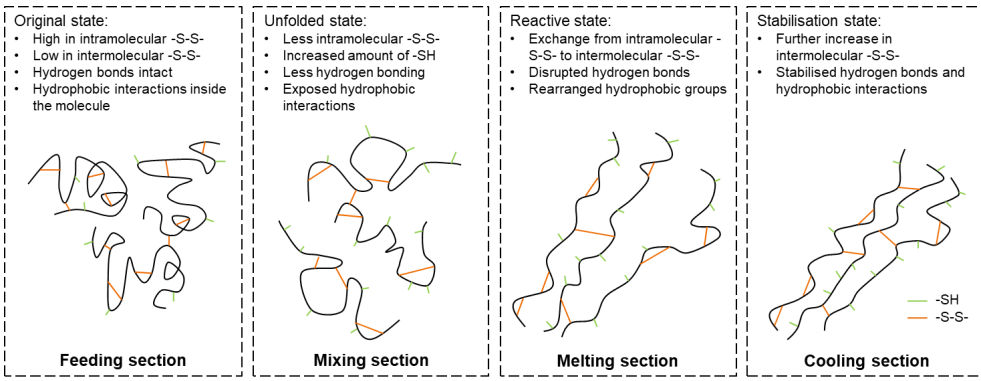


Figure 4. Rough illustration of protein interactions during HMEP based on the findings reported by (Cornet et al., 2022; Liu and Hsieh, 2008; Zhang et al., 2022b). Covalent interactions, including intra- and intermolecular disulphide bonds (-S-S-) as well as free thiol groups (-SH) are illustrated in the drawing, while non-covalent interactions, including hydrogen bonds and hydrophobic interaction are only explained in the text.

Several studies have investigated the transformations in the secondary structures (α-helices, β-sheets, β-turns, and random coils) of varying plant proteins during HMEP, across the feeding section to the successfully aligned fibrous structure (Dou et al., 2022; Meng et al., 2022; Peng et al., 2022; Wang et al., 2023a; Zhang et al., 2021b; Zhang et al., 2022b). Table 10 summarises the observed trends from the feeding or mixing zone through to the cooling die or fibrous extrudate. While the trends exhibited slight variations depending on the protein source, certain consistent patters were apparent, particularly within the same protein sources. For instance, all studies, except for those focusing on peanut proteins, noted an increase in β-sheet structures during the process. Additionally, there was a trend of increasing content of α-helices, except for pea protein isolate, however, none of the changes were significant. In the majority of the studies, β-turns, and random coil structures decreased. However, the significance of these changes varied across the studies.

Table 10. Transformation of secondary structures of plant proteins derived from peanut, pea, soy, and wheat during high-moisture extrusion processing.

Raw material	α-helix	β-sheet	β-turn	Random coil
Peanut protein [a]	↗	↘*	↗*	↘
Pea protein isolate [b]	(↘)	↗	↘*	(↗)
SPC/WG (50:50) [c]	↗	↗*	↘*	↘*
Soy protein isolate [d]	↗	↗*	↘	↘*
Soy protein concentrate [e]	↗	↗	↘	↗
SPI/WG (70:30) [f]	(-)	↗	↗*	↘*

The arrows indicate whether the specific structure experienced an increase ↗ or decrease ↘ during the transition from feeder section to cooling section / extrudate. The brackets (-) around the dashes indicate for minute change. The asterisk * indicates a significant change.

Abbreviations: SPC = soy protein concentrate, SPI = soy protein isolate, and WG = wheat gluten.

References: [a] Zhang et al. (2021b), [b] Peng et al. (2022), [c] Zhang et al. (2022b), [d] Meng et al. (2022), [e] Dou et al. (2022), [f] Wang et al. (2023a)

Another common finding across these studies was the notable fluctuation in counts of secondary structures during the intermediate melting section, which, by the time of reaching cooling zone, returned to levels closer those of the feeding section. For instance, in peanut protein, the initial count of 38% β-sheets increased to 47% in the melting section, decreased to 36% in the cooling section and finally settled to 30% in the extrudate. Similarly, the random coil structure in soy protein concentrate, initially at 23%, declined to 19% in the melting and cooling zones, and then rose to 24% in the fibrous extrudate, a level not significantly different from its original count. These observations suggest that during the melting section of HMEP, protein structures unfold and subsequently rearrange into a different conformation showing similar or different ratios of secondary structures compared to their original state.

2.3.4 Impact of non-protein compounds on fibrous structures

Plant protein ingredients, particularly those produced on an industrial scale with a focus on feasibility and minimal environmental impact (such as dry fractionated ingredients), are rarely composed of solely isolated proteins. Therefore, it is essential to understand the interactions between proteins and other components, such as starch, dietary fibre, and other polysaccharides, as well as fats and oils. However, the mechanism of these interac-

tions is less understood than that of protein-protein interactions. The existing limited research on this topic has focused on investigating the maximum inclusion rates of these substances.

For instance, Kendler et al. (2021) reported that increased levels of medium-chain glycerides, a type of saturated fat in coconut, reduced the anisotropy of wheat protein extrudates. Interestingly, they observed that by adding the fat directly to the melting section, rather than the mixing section, fibrous structure remained more pronounced with smaller oil droplets in microscopic level. In further studies by Chen et al. (2023), saturated fatty acids were added during extrusion of pea protein isolate, which increased the disulfide bond formation, evidenced as enhanced fibrous structures. However, increased saturation degrees of the added fatty acids disrupted the fibrous structure by promoting the hydrophobic interactions instead of the disulphide bonding. Microscopic inspection showed that saturated fatty acids distributed uniformly in small spots, while unsaturated fatty acids formed large, unevenly distributed oil clusters. Additionally, the decrease in free amino ends suggested that unsaturated fatty acids formed complexes with proteins.

The incorporation of polysaccharides, namely sodium alginate, xanthan gum, maltodextrin, iota carrageenan, and carboxymethyl cellulose sodium, has been demonstrated to enhance the formation of fibrous structures in soy proteins and gluten-based meat analogues during HMEP (Dou et al., 2022; Wang et al., 2023a). Despite the observed increase in hydrogen bonding levels, as reported by Dou et al., (2022) and Wang et al. (2023a), a comprehensive understanding of the molecular-level mechanisms, particularly the interactions between proteins and polysaccharides, remains unclear.

In the context of starch inclusion, Bühler et al. (2022) identified an optimal inclusion rate, that improves the fibrous structure of gluten in shear cell structuring. Beyond this optimal point, the inclusion of starch begins to negatively impact the anisotropy of the structure. The extent of disruption was found to be dependent on the type of starch used. For instance, starch composed of 1% amylose was observed to negatively affect the structure even at low inclusion rates. Conversely, starches with higher amylose content, specifically 25% and 68%, were able to maintain the structure up to relatively high inclusion rates of 30-40%. Furthermore, pre-gelatinisation of starch through heat treatment was found to induce the formation of fibrous structures in samples that were otherwise incapable of forming fibres. Again, the mechanism of this phenomenon remained unsolved. On the other hand, Chen et al. (2022) discovered that amylopectin was more effective than amylose in enhancing the formation of fibrous structures in pea protein isolate.

The integration of insoluble dietary fibre to soy protein isolate has been shown to significantly enhance the fibrous structure up to and inclusion rate of 20%, while simultaneously lightening the colour of the extrudate (Deng et al., 2023). This partial substitution of soy protein isolate with insoluble dietary fibre resulted in an enhanced mechanical anisotropy. This enhancement was attributed to the disruption of the continuous matrix of soy proteins into filaments. These filaments, observable at the microscopic level and aligned with the direction of extrusion flow, resulted in a decrease in the hardness, springiness, and chewiness of the final product.

2.3.5 Functionalisation strategies for improved fibrous structures

The majority of the existing research on meat analogues focuses on investigating the process parameters and ingredient blending for achieving fibrous structures. However, it is also important to understand whether certain pre-treatments (that could be conducted already at manufacturing phase of the raw materials and ingredients) can enhance or induce the formation of meat-like fibrous structures. This is particularly relevant for ingredients with low protein content, which are often more sustainable and feasible compared to the isolates. However, the research in this area has remained limited. Therefore, this section aims to introduce the limited research conducted on functionalisation strategies designed to improve the ability of plant protein ingredients to form fibrous structures during HMEP.

One approach involves pre-toasting of soy proteins in oven as a part of their fractionation process (Geerts et al., 2018). The implementation of this strategy resulted in an improved fibrous structure formation after toasting treatment at 150 °C. The improved structure formation was accompanied with the improved water-holding capacity and limited protein solubility of the ingredient. Interestingly, higher toasting temperatures resulted in complete protein denaturation and formed inadequate sandy structures were observed.

In another study, transglutaminase was introduced directly into the extruder along with the protein ingredient through the feeder (Zhang et al., 2021b; Zhang et al., 2023). They reported increased values for fibrous degrees in soy and pea protein isolates, while a decrease was noted in soy protein concentrate. The change in fibrous degree of peanut protein was found to be minute although they reported higher values in their earlier study. Although they were not able to quantify the fibrous degree for wheat gluten and rice protein isolate due to their inconsistent structures, clear fibres were visible in wheat gluten prior to the transglutaminase treatment. Conversely, rice protein isolate formed crumbly structures, regardless of the presence or absence of transglutaminase.

Peng et al. (2022) investigated the influence of L-cysteine addition (0-0.15 %) on fibrous structure formation of pea protein isolate during HMEP. They reported an increasing trend in fibrous degree, hardness, and springiness values until a concentration of 0.09%. However, beyond this threshold, the addition of cysteine exhibited a negative effect on the fibrous structure. Despite these structural changes, no statistically significant alteration in protein secondary structures.

In conclusion, the functionalisation methods discussed above, including the addition of cysteine and transglutaminase as well as pre-heating, have demonstrated their potential to enhance fibrous structure formation at least to a certain extent. Despite these promising results, significant limitations persist depending on the protein source. Particularly low-protein ingredients have received less attention. Therefore, further research is needed in order to explore more functionalisation strategies, introduced in section 2.2, for these underperforming ingredients, which, despite their current limitations, offer sustainable and healthy alternatives for meat analogue application.

3. Aims of the study

The objective of this study was to tackle the challenges associated with the poor functionality of plant proteins to improve their performance in food applications. This study concentrated on two primary objectives: enhancing the functionality of insoluble wet-extracted material with high protein content for solubility-dependent applications, and developing methods to improve the fibrous structure formation of plant proteins for use in meat analogues

The initial focus was on rice protein isolate, a by-product of starch production, generated through wet extraction. The utilised strategy involved functionalisation through limited enzymatic hydrolysis to improve its near-zero solubility and other functional properties. The hypothesis was that controlled enzymatic protein hydrolysis could reduce the molecular weight of heavily denatured and aggregated rice proteins, thereby increasing their solubility and ultimately their functional properties.

Following this, the focus shifted towards meat analogue applications. The investigation extended beyond the challenging rice protein ingredient to include a significant emphasis on dry fractionated low-protein ingredients including pea and oat, protein concentrates. These ingredients pose a particular challenge when it comes to structuring for meat analogue applications due to their low protein content. Therefore, the objective was to overcome these challenges by improving their fibrous structure formation ability using chemical and enzymatic functionalisation strategies. Chemical pH-shift was employed to understand the effect of pH on the formation of fibrous structures of several plant sources with varying protein contents, including the rice protein isolate, pea protein concentrate, pea protein isolate, and wheat gluten. As functional properties are usually increased at higher pH levels, it was hypothesised that pH-shifting could also improve the fibrous structure formation. The enzymatic functionalisation approach involved crosslinking and deamidation of oat protein concentrate, known for its high starch content. As disulphide bonding is identified as one of the key reactions involved in fibrous structure formation, it was hypothesised that enzymatically created crosslinks would facilitate the formation of protein networks, despite the presence of high levels of interfering starch. Finally, given that rapeseed protein concentrate emerged as a promising low-protein ingredient capable of forming an acceptable fibrous structure, the final aim was to address its significant sensorial challenges using fermentation as a functionalisation strategy.

This multifaceted approach allowed us to address the various challenges associated with different types of plant proteins and their application in meat analogues and solubility-dependent applications.

4. Materials and methods

4.1 Raw materials and enzymes

This study investigated three different protein concentrates derived from rapeseed, yellow pea, and oats as well as three different protein isolates derived from rice, yellow pea, and wheat (Table 11). Rapeseed protein concentrate (RPC) was produced at VTT from a rapeseed press cake provided by Kankaisten Öljykasvit (Turenki, Finland). The press cake was fat-extracted using supercritical carbon dioxide (SC-CO_2) prior to milling and air-classification described in publication IV. Oat protein concentrate (OPC) was also produced by milling and air-classification from dehulled oat kernels described in publication II. Commercial dry fractionated pea protein concentrate (PPC) was provided by Vestkorn Milling AS, which was also produced by milling and air-classification as is described at the product website. Rice endosperm protein isolate (REPI) was provided by Beneo/Südzucker, and it was produced from a side-stream of starch production using pH-shifting and subsequent precipitation of the proteins at their isoelectric point. Pea protein isolate (PPI) is produced by wet extraction as well. Wheat gluten (WG) was provided by Beneo GmbH, and the production method is described at their website using the traditional method for gluten extraction.

Table 11. Overview of the raw materials used in this study, their abbreviation, manufacturing description, and supplier information.

Raw material	Abbreviation	Description	Producer	Publication
Pea protein concentrate	PPC	Dry fractionated, commercial	Vestkorn Milling AS	II
Rapeseed protein concentrate	RPC	Dry fractionated, experimental	VTT	IV
Oat protein concentrate	OPC	Dry fractionated, experimental	VTT	III
Rice endosperm protein	REPI	Wet-extracted, commercial	Beneo	I, II
Pea protein isolate	PPI	Wet-extracted, commercial	Roquette	II
Wheat gluten	WG	Wet-extracted, commercial	Beneo	II

Table 12 shows the enzymes and microbial strains employed in this study for the raw material functionalisation. Neutral and acid endoproteases were used to hydrolyse the REPI. In contrast, transglutaminase along with combination of trans- and protein-glutaminase, were utilised for the crosslinking and deamidation the OPC. Additionally, this study used two different microbial strains for the fermentation of RPC from VTT culture collection, namely *Lactiplantibacillus plantarum* (E-78076) and *Weissella confusa* (E-143403).

Table 12. Overview of the enzymes and microbial strains used in this study including their description, manufacturer, main activity, functionalised raw material, and the publication number in Roman numbers. REPI = rice protein isolate, OPC = oat protein concentrate, RPC = rapeseed concentrate.

Enzyme / Microbial strain	Description	Manufacturer	Main activity (U/g)	Raw material
Neutral endoprotease	Experimental	AB Enzymes, Germany	1000	REPI (I)
Acid endoprotease	Experimental	AB Enzymes, Germany	453	REPI (I)
Transglutaminase	Commercial	Ajinomoto Inc., Japan	100	OPC (III)
Trans-/protein-glutaminase	Commercial	Ajinomoto Inc., Japan	100/150	OPC (III)
α-amylase	Commercial	Novozymes, Denmark	480	OPC (III)
Lactiplantibacillus plantarum	E-78076	VTT culture collection	-	RPC (IV)
Weissella confusa	E-143403	VTT culture collection	-	RPC (IV)

4.2 Overview of the experimental research

As this study investigated a wide range of functionalisation strategies of various plant proteins, a comprehensive overview of the experimental design is provided in Figure 5. Limited enzymatic hydrolysis was investigated to enhance the functionality of REPI, specifically for application dependent on protein solubility. These applications included foaming, colloidal stability, and gelation. Chemical pH shift to 5 or 7 was employed to enhance the fibrous structure formation of REPI, WG, PPI, and PPC in HMEP. Furthermore, enzymatic deamidation and crosslinking was utilised to enhance the fibrous structure formation of OPC with and without a pre-heating step (and with or without α-amylase treatment). Finally, fermentation combined with pH-shifting was investigated in relation to sensory properties and fibrous structure formation of RPC.

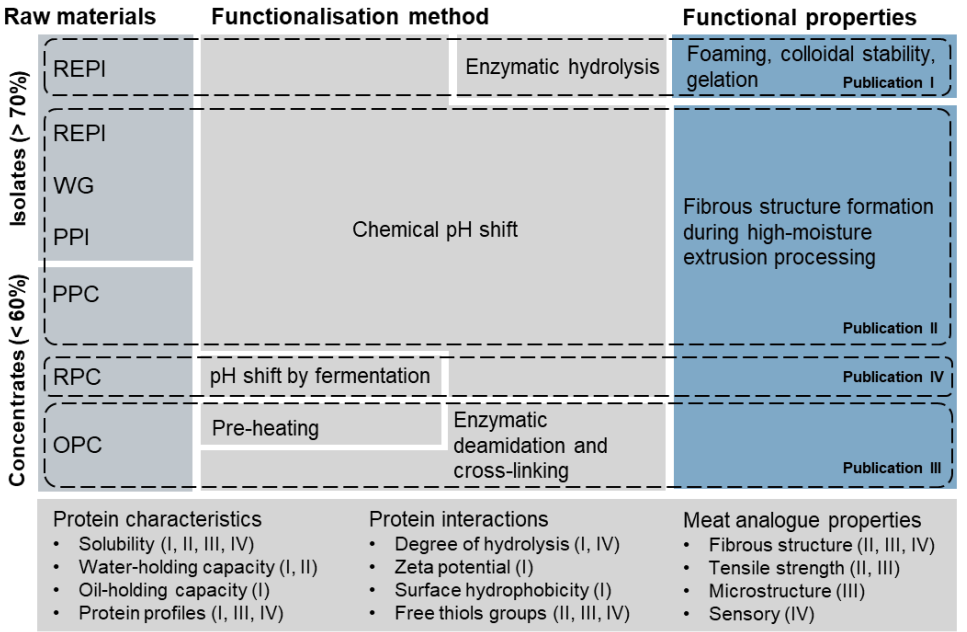


Figure 5. Overview of the experimental design of this study including the raw materials, functionalisation strategies, functional properties, and other analytics. REPI = rice endosperm protein isolate, WG = wheat gluten, PPI = pea protein isolate, PPC = pea protein concentrate, RPC = rapeseed protein concentrate, OPC = oat protein concentrate.

4.3 Functionalisation

4.3.1 Enzymatic modification

REPI was hydrolysed by neutral and acid endoproteases under controlled conditions (I). It was dispersed in milli-Q water at a concentration of 10%, w/V, and the pH was adjusted to either 5 or 7 according to the used endoprotease. The hydrolysis and control reactions were carried out at 40 °C with the pH maintained constant throughout the 3-hour treatment. Endoprotease dosages of 0.0 (control), 0.2, 2.0 and 20 U/g of substrate protein were used. Aliquots for different analyses were collected from the suspensions, which included both soluble and insoluble substances, and immediately frozen to minimise enzyme activity.

Enzymatic treatments for OPC were conducted in two stages (III). Initial trials were performed on a smaller scale using 50 ml volume with a 4% (w/w) OPC concentration. This was later scaled up to 1.5 litres, using an OPC concentration of 30% (w/w). Both trials were conducted using distilled water without adjusting the pH during the treatments. Two different approaches were used prior to enzymatic modification; one involved a pre-heating step at 95 °C for 15 min, and the other did not involve heating with the enzyme dosed directly. Following this, pre-heated suspensions were cooled to 40 °C, and non-heated suspensions were tempered to the same temperature. Subsequently, crosslinking (using transglutaminase) and combined crosslinking with deamidation (using a combination of trans- and protein-glutaminase) reactions were carried out. These treatments were maintained at 40 °C for 2 hours (or 4 hours in the 50 ml scale) under constant stirring, using enzyme dosages of 0.0 (control), 0.5 or 5.0 U/g of OPC. Additionally, starch degradation treatments were conducted by adding α-amylase to all pre-heated samples using either 0 U dosage (for pre-heated control with an addition of 2 mg/g amylase) or 5.0 U dosage of crosslinking and deamidating enzymes with addition of 2 mg/g amylase. After the incubation, the suspensions were immediately frozen to suspend the enzyme activity, then freeze-dried, and finally pulverised using a kitchen cutter. Raw material characteristics (solubility, particle size, and free thiol groups were measured from the samples in 50 ml scale while the extrudate characteristics were analysed from the 1.5 l scale samples.

4.3.2 Fermentation

Fermentation was studied both as an independent treatment and in combination with pH-shifting as a functionalisation strategy for RPC prior to HMEP (IV). Additionally, an extensive range of control treatments employing pH-shifting were conducted. Table 13 summarises all treatments conducted on RPC.

Fermentation and combined pH-shifting
The used strains, *L. plantarum and W. confusa*, were cultivated on a General Edible medium. RPC was dispersed at a concentration of 25% (w/w) in sterile distilled deionised water, combined with 1% inoculum, and incubated at 25 °C for 24 h. A modification was made to one *L. plantarum* sample by adding a step to shift the pH back to its original value of 6. Following incubation, the dispersions were immediately frozen, freeze-dried, and pulverised using a kitchen cutter.

Control treatments employing pH-shifting

Three distinct control treatments were conducted, each achieving a final pH of around 4. In parallel, three additional control treatments were prepared, each with a final pH of around 6. The first control sample was adjusted to pH 4 using HCl, while the second one used lactic acid for the same purpose. Additionally, the third control treatment was adjusted to pH 4 using lactic followed by an incubation step that replicated the conditions employed during fermentation (25 °C for 24 h). Additionally, three control treatments were prepared in a manner similar to the first three samples, but with an intended final pH of 6. This was achieved by adding an extra step of adjusting the pH to 6 using NaOH. Finally, all samples were frozen, freeze-dried, and pulverised.

Table 13. Summary of preparation steps of fermented and control rapeseed protein concentrate samples.

Final pH target	1. pH shift to 4	Incubation step	2. pH shift to 6	Abbreviation
4	*L. plantarum*	25 °C, 24h	-	f-LP-pH4
5	*W. confusa*	25 °C, 24h	-	f-WC-pH4
6	*L. plantarum*	25 °C, 24h	NaOH	f-LP-pH6
4	HCl	-	-	h-pH4
4	Lactic acid	-	-	la-pH4
4	Lactic acid	25 °C, 24h	-	i-la-pH4
6	HCl	-	NaOH	h-pH6
6	Lactic acid	-	NaOH	la-pH6
6	Lactic acid	25 °C, 24h	NaOH	i-la-pH6
6	-	-	-	Native RPC

Abbreviations: RPC = rapeseed protein concentrate, LP = *Lactiplantibacillus plantarum*, WC = *Weissella confusa*, f = fermented, h = HCl, la = lactic acid, i = incubated.

4.3.3 Chemical pH-shifting

This study investigated the use of pH-shifting on PPC, PPI, REPI, and WG as a method to improve fibrous structure in HMEP. The raw materials were suspended in tap water at a concentration of 20% (w/w) and were shifted to pH levels of 5, 7, and 8. The pH adjustment was conducted under constant stirring for 2 hours using either 6 M or 1 M HCl or NaOH. The control treatments were prepared identically, but without the pH adjustment. Following this, the suspensions were immediately frozen and freeze-dried for subsequent application studies. The pH-shifted REPI, PPC, and PPI at three different pH values were pulverised in a kitchen cutter, while WG samples required additional milling using a lab-scale centrifugal mill (Retsch, Germany) equipped with 0.5 mm sieve at 12 000 rpm.

4.4 High-moisture extrusion processing

This study investigated the functionalised ingredients in high-moisture extrusion processing employing a co-rotating twin-screw extruder (Process 11 Hygienic, Thermo Scientific, Karlsruhe, Germany) equipped with a long slit cooling die (5 × 20 × 250 mm, H × W × L). The extruder screws, with a length of 440 mm and a diameter of 11 mm, corresponded to a length-to-diameter (L:D) ratio of 40:1. Table 14 provides a detailed overview of the operational and monitored extrusion parameters for each sample during the processing runs (II, III, IV). These parameters include the temperature profile within the barrel, the cooling die temperature, the melt temperature of the protein inside the die body just prior to entering the cooling die, as well as the flour feed, water feed, and screw speed. Additionally, the table includes monitored extruder responses such as motor torque and pressure at die body.

Table 14. Operational parameters in high-moisture extrusion processing of the functionalised raw materials along with the extruder responses in publication II, III, and IV.

Raw material	Sample	pH	Melt T (°C)	Temperature profile from feeder to die (°C)	Cooling die T (°C)	Flour feed (g/h)	Water feed (ml/h)	Screw speed (rpm)	Torque (Nm)	Pressure at die (bar)
REPI		5	95	100-100-110-110-100-80-70-60	30	280	313	350	1.4	38
(II)			140	143-145-160-160-140-80-70-60	30	280	313	350	1.3-1.6	31-32
			160	164-165-170-170-140-80-70-60	30	280	313	350	1.9-2.2	44-62
		7	95	100-105-110-110-90-80-70-60	30	250	303	300	1.3	30
			140	142-145-160-160-140-80-70-60	30	280	313	350	2.1-1.9	48-66
			160	162-165-170-170-140-80-70-60	30	280	313	350	1.8-2.1	68-83
WG		5	95	97-110-110-100-90-80-70-60	45	250	274	250	0.8	7-8
(II)			140	145-150-150-110-90-80-70-60	45	250	274	250	0.8	8
			160	170-180-180-130-90-80-70-60	45	250	274	250	0.8	3-11
		7	95	97-110-110-100-90-80-70-60	45	190	254	200	0.8	8
			140	145-150-150-110-90-80-70-60	45	180	194	250	0.8-1.2	10-37
			160	168-180-180-130-90-80-70-60	45	180	194	250	0.7-0.8	8-9
PPC		5	95	96-108-108-110-90-80-70-60	30	280	284	300	1.5-1.6	36-46
(II)			140	143-158-160-130-90-80-70-60	30	250	254	300	1.2-1.3	23-31
			160	168-170-170-140-90-80-70-60	30	50	254	300	0.9-1.0	11-14
		7	95	97-108-108-110-90-80-70-60	30	280	284	300	1.4	32-38
			140	146-155-155-130-90-80-70-60	30	250	254	300	1	14-16
			160	168-175-170-140-90-80-70-60	30	250	254	300	0.9	12-14
PPI		5	95	98-95-95-95-90-80-70-60	30	280	314	300	1.2-1.4	31-40
(II)			115	118-130-130-100-90-80-70-60	30	200	264	200	0.9-1.0	21-22
			135	137-159-160-130-100-80-70-60	30	190	254	200	1.3	36-39
		7	95	98-95-95-95-90-80-70-60	30	280	313	300	1.2	31-38
			115	117-125-125-1115-90-80-70-60	30	280	313	200	1.3-1.4	35-42
			135	136-159-160-130-100-80-70-60	30	190	254	200	0.6	5-6
OPC	Native	6.4	-	155-165-165-140-120-90-70-60	30	0.25	204	300	0.9	4
(III)	Control	6.3	152	160-160-160-140-120-90-80-70	30	0.3	257	300	0.9	9
	H-control	6.5	157	165-170-170-145-120-90-80-70	30	0.3	256	300	0.9	8
	TPG 0.5U	6.5	154	160-165-165-140-110-90-80-70	30	0.3	257	400	1.0	10
	TPG 5U	6.6	158	165-165-165-140-120-90-80-70	30	0.3	257	400	1.0	13
	H-TPG 5U	6.8	159	165-175-175-145-120-90-80-70	30	0.3	256	400	1.3	19
	TG 0.5U	6.3	154	160-170-170-140-120-90-80-70	30	0.3	247	400	1.0	11
	TG 5U	6.3	158	165-170-170-145-120-90-80-70	30	0.3	236	300	1.0	5
	H-TG 5U	6.5	163	170-175-175-145-120-90-80-70	30	0.3	226	300	0.9	9
RPC	Native	6.0	142	65-70-80-130-160-160-165-145	30	300	304	200	0.8	7
(IV)	f-LP	4.2	149	60-70-80-95-150-160-160-152	30	250	185	300	1	15
	f-WC	5.2	160	60-70-80-95-150-175-175-165	30	250	185	300	0.9	12
	f-LP	6.0	146	60-70-80-110-130-160-165-149	30	270	257	300	1	12
	h	4.1	148	60-70-70-90-120-150-165-160	30	250	215	250	0.8	9
	la	4.1	146	60-70-80-110-130-160-165-149	30	270	244	200	1	16
	i-la	3.8	146	60-70-80-110-130-160-165-147	30	270	244	200	0.7	7
	h	6.1	146	60-70-80-110-130-160-165-149	30	270	267	300	1	10
	la	6.1	145	60-70-80-110-130-160-165-149	30	270	267	300	1.2	18
	i-la	6.1	146	60-70-80-110-130-160-165-149	30	270	257	300	0.9	11

Abbreviations: REPI = rice endosperm protein isolate, WG = wheat gluten, PPC = pea protein concentrate, PPI= pea protein isolate, OPC = oat protein concentrate, RPC= rapeseed protein concentrate, T = temperature, H = pre-heated, TG = transglutaminase, TPG = combined trans and protein-glutaminase, U = enzyme dosage in units, f= fermented, LP = *Lactiplantibacillus plantarum*, WC = *Weissella confusa*, i = incubated, h = HCl, la = lactic acid.

In the starting point of all HMEP trials, the initial barrel setting for temperature profile was 90-90-90-90-80-80-70-60 to prevent die blockage and sample burning of. The initial water content was set slightly higher than the feed rate to ensure a smooth transition of the material to cooling die. The starting screw speed was typically 300 rpm and was adjusted according to each raw material behaviour to achieve a stable flow. Gradually, the temperature profile was increased, and necessary adjustments were made to the water feed until fibrous or rigid (in case none of the temperature lead to fibrillation) structure was observed.

4.5 Analytical methods

4.5.1 Composition

The protein content was analysed using a Kjeldahl /Dumas autoanalyser (Foss Tecator Ab, Sweden), applying a nitrogen conversion factor of 5.95 (I) or 6.25 (II, III, IV). The total starch content (II) was measured according to AOAC 2002.02 method using a Megazyme resistant starch assay kit (K-RSTAR 08/18). The total lipid content (III) was determined as the sum of individual fatty acids as described in publication III. The ash content (III) was measured gravimetrically after combusting of the raw materials at 550 °C for 41 hours in a muffle furnace (model N11, Nabertherm GmbH, Lilienthal/Bremen, Germany). The moisture content (I, II, IV) was analysed gravimetrically following the drying of the raw materials at 105 °C for 24 h.

4.5.2 Degree of hydrolysis

The degree of hydrolysis (I, IV) was calculated according to the following equation as the percentage of cleaved peptide bonds (h) to the ratio of total peptides bonds (h_{tot}) in the raw materials as described in detail in publication I:

$$DH = \frac{h}{h_{tot}} \cdot 100\% \quad (1)$$

The cleaved peptide bonds were measured using an OPA/NAC (ortho-phthalaldehyde/*N*-acetyl-L-cysteine) reagent, which was prepared according to Spellman et al. (2003).

4.6 Functional and physicochemical properties

Protein solubility
The protein solubility (I, II, III, IV) of proteins in water was calculated as the percentage of solubilised proteins in ratio to the total protein content in the suspension. The solubilised proteins were measured using the Kjeldahl/Dumas combustion method (I, II, III, IV) or a colorimetric Bio-Rad DC protein assay kit (Bio-Rad Laboratories Inc., USA) (III). Further details on sample preparations are described in the publications. It is worth mentioning that in publication I, the used centrifugation forces were relatively low (3000 g). Therefore, the protein solubility might also include molecules that are not fully soluble, but in a colloidally stable form.

Particle size
The particle size distributions were measured in publications II and III using laser diffraction method with a Malvern Mastersizer 3000 machine (Malver Instruments, Worcestershire, UK). The machine was equipped with Hydro LV liquid dispersion unit, except for WG, the Aero S dry dispersion unit was utilised due to the formation of gluten aggregates in the presence of water.

Foaming, colloidal stability, gelation, surface hydrophobicity, and zeta potential
The 10% suspension concentration used for producing the samples was chosen based on preliminary trials and the similar dry matter concentration to egg white. This ensured the results were comparable to the foaming and gelation properties of egg white. The foaming properties, colloidal stability, gel formation, surface hydrophobicity, and zeta potential of

the REPI samples were investigated in publication I. The frozen sample aliquots for each analysis were thawed prior to the analysis. Foaming was induced by whipping the sample in measuring cylinder using a battery operates whisk (Aero Latte AL-V1-SS Chef Kitchen Whisk, UK) until the foam height reached a plateau. Foam capacity, drainage and foam stability were monitored and calculated according to the detailed description and equations in publication I. Colloidal stability was analysed as a function of time using a glass tube to monitor the clarification of the top of the protein suspension. The clarified part in relation to the opaque part was analysed from digital images taken at certain time intervals. The detailed equations for calculating the percentage value of colloidal stability are described in detail in publication I. Gel formation ability of REPI samples was analysed in Eppendorf tube using tilt-tube method. Shortly, the samples were heated at 98 °C, stored at 4 °C for 24 h and tilted to see if the gels collapsed. The surface hydrophobicity was analysed from the supernatants of REPI samples with 1-anilino-8-napthalene (ANS) probe using fluorescence spectrometer (Varioskan, Thermo Fisher Scientific, Germany). REPI samples were diluted to a protein concentration range of 0.005 to 0.5 mg/ml with pH 5 or pH 7 buffers, and subsequently mixed with ANS reagent. Fluorescence intensity was measured using excitation and emission wavelengths of 390 and 479 nm, respectively. The degree of electrostatic repulsion of the solubilised proteins in the supernatants of REPI samples were analysed as zeta potential values using Zetasizer Nano ZS equipment (Malvern Instruments Ltd., UK).

Water and oil-holding capacities

The water-holding capacity of protein samples was analysed in publications I and II. The protein raw material was mixed with mQ-water, vortexed at 0, 5, and 10 min intervals, and centrifuged at 750 × g for 15 min at 20 °C. The supernatant was then separated by pouring. The water-holding capacity was calculated as the gain in weight per original weight. The oil-holding capacity, analysed only in publication I, was analysed similarly, but using sunflower oil instead of water.

Viscosity

The viscosity profiles of the OPC samples were analysed in publication III using a Rapid Visco Analyser (RVA) super 4 (Newport Scientific, Australia). A 10% (w/w) solution was prepared and run under constant stirring (160 rpm) using the following temperature program: 1) temperature increase from 25 °C to 95 °C at 10 °C/min speed, 2) mixing at 95 °C for 15 min followed by cooling to 25 °C at 10 °C/min speed.

Protein profiles

The molecular weight distributions of the proteins in REPI, OPC and RPC samples were analysed using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as described in publications I, III and IV, respectively. All publications used Criterion TGX stain-free precast gels (Bio-Rad Hercules, CA, USA) with a commercial standard ranging from 10 to 250 kDa (Precision Plus Protein Unstained standards, Bio-Rad, Hercules, CA, USA). Reducing sample buffer (20% (v/v) glycerol, 4% (w/v) SDS, 10% (v/v) β- mercaptoethanol and 0.02% (w/v) bromophenol blue in 0.1M Tris-HCl, pH 6.8) was used in publications I, III, IV while non-reducing sample buffer (no β-mercaptoethanol) was used only in publications III and IV. The gels were visualised using a Criterion stain-free imaging system (Bio-Rad, Hercules, CA, USA).

4.7 Structure-related properties

4.7.1 Macro and microstructures

The macrostructures of the samples and extrudates were captured using a mobile phone camera (I, II, III, IV). Microstructures of the extrudates, analysed only in publication II, were analysed with a Zeiss LSM 710 confocal laser-scanning microscope. The extrudates were cut at a 45° angle and stained with Acid Fuchsin (BSH Chemicals Ltd., Poole, Dorset, UK) for examination. A HeNe laser line of 543 nm was used for excitation, with emission collected at 538-703 nm. The final micrographs, where the protein appears red, were assembled as maximum intensity projections. Representative images were selected for each sample.

4.7.2 Tensile strength

The tensile strength of the extrudates (II, III) was measured according to Nisov et al. (2020a) with slight modifications. It analysed using a Lloyd LS5 material testing machine (Ametek, Berwyn, PA, USA) with test speed of 2 mm/min. The extrudate sizes during the measurements were 30 × 20 × 5 mm and 20 × 20 × 5 mm (L × W × H) in publications II and III, respectively. The width and height were determined by the size of the extrudate strip as it emerged from the cooling die, while the length was manually cut. In publication II, the 30 mm length was used to be able to ensure that the extrudate didn't slip from the jaws during the tensile measurement. In subsequent studies, it was demonstrated that the jaws could securely hold a shorter extrudate, and that these samples yielded similar values.

4.7.3 Free thiol groups

The free thiol groups of the raw materials and extrudates were studied in all HMEP-related publications (II, III, IV). Prior to analysis, all sample powders and extrudates were similarly milled using a lab-scale ball-mill (MM301 Mixer Mill, Retsch, Germany). The samples were analysed using a method based on Ellman's reagent according to the details described in Nisov et al. (2020a) and publications II, III, and IV. Publication III investigated free thiol groups with reducing (non-covalent interaction broken by urea addition) and non-reducing buffers while publications II and III used only non-reducing buffer.

4.8 Cysteine content

Cysteine (and methionine) were determined as cystic acid (and methionine sulfone) after an oxidation procedure in total amino acid analysis. Briefly, for the analysis of the total content of amino acids, dry samples (5 mg) were accurately weighed. Subsequently, the samples were oxidized with a fresh performic acid solution and then hydrolysed using hydrochloric acid. The derivatisation and ultra-performance liquid chromatography analysis of the samples were performed according to Waters (Milford, MA, USA) AccQ-Tag method. Quantification was based on calibration curves of reference substances. Three replicate derivatisations and ultra-performance liquid chromatography runs were conducted from each sample hydrolysate.

4.9 Sensory profile

The sensory properties of the modified RPC samples (IV) were evaluated using the generic descriptive method by a trained sensory panel. The panel was composed of 6-7 assessors, a size determined by the restrictions prevailing during the pandemic times of COVID-19. A total of 13 distinct attributes involving aspects related to flavour, appearance and structure, were evaluated, as detailed in the publication IV.

4.10 Statistical analysis

Statistical analysis was carried out using the SPSS Statistics software. Generally, a one-way analysis of variance (ANOVA) was employed, accompanied by Tukey's HSD as the post hoc test (II, III, IV). In instances where the homogeneity of variance was violated, Games Howell or Tukey's Honestly Significant Difference (HSD) were used as the post hoc tests. Additionally, when comparing the behaviour of two distinct groups (extrudates versus raw materials), an independent samples T-Test with Bonferroni correction was employed (IV). Sensory data was analysed using a two-way mixed model ANOVA, with samples as the fixed factor and assessors as a random factor. Tukey's HSD was used as the post hoc test in the sensory-related analyses. The limit for statistical significance was set at $p < 0.05$.

5. Results and discussion

5.1 Composition and physicochemical properties of raw materials

This study involved six protein-enriched raw materials derived from five distinct plant sources (rice, wheat, pea, rapeseed, and oat) produced through both dry and wet fractionation processes, resulting in varying compositions (Table 15). In compositional analysis, the wet-fractionated raw materials (REPI, WG, and PPI) had over 70% protein contents, thus, classifying them as protein isolates. In contrast, the dry fractionated PPC, RPC and OPC showed protein contents ranging from 39.5% to 48.5 %, grouping them as protein concentrates. The OPC exhibited substantial starch content of 33.0%, distinguishing itself from the other raw materials, which contained less than 10% starch. Notably, PPI and RPC exhibited nearly zero starch content. All raw materials demonstrated less than 4.5% lipid content and contained similar amounts of moisture ranging from 6.2 to 7.8%. Raw materials derived from pea and rapeseed exhibited clearly higher values of ash (3.8-9.0%) compared to those from rice and wheat (~1%). This was in accordance with the higher amount of ash found in rapeseed and pea seeds compared to rice and wheat (Amagliani et al., 2017; Belitz et al., 2009; Nadathur et al., 2017; Rommi et al., 2014).

Table 15. Chemical composition of rice endosperm protein, wheat gluten, pea protein isolate, pea protein concentrate, rapeseed protein concentrate, and oat protein concentrate.

Abbreviation	Protein (%)	Starch (%)	Lipids (%)	Ash (%)	Moisture (%)	Publication
REPI	72.0	9.2	2.4	1.0	7.4	I, II
WG	73.1	9.4	4.1	1.0	7.8	II
PPI	74.4	0.4	4.6	3.8	6.6	II
PPC	48.5	6.1	2.5	5.7	7.2	II
OPC	39.5	33	<2.5*	na	na	III
RPC	41.1	na	2.8	9.1**	6.2	IV

* Estimate based on Sibakov et al. (2011), ** estimate based on Silventoinen et al. (2022)

Abbreviations: REPI = rice endosperm protein, WG = wheat gluten, PPI = pea protein isolates, PPC = pea protein concentrate, OPC = oat protein concentrate, RPC = rapeseed protein concentrate, na = not analysed

The studied raw materials were characterised for pH, conductivity, solubility, water-holding capacity, and particle size (Table 16) to facilitate the selection of functionalisation strategies. The original pH of the raw materials in water-suspension varied from 4.5 to 7.9, which can greatly affect the measured properties in Table 16, including solubility and particle size (Peng et al., 2020). Dry-fractionated raw materials exhibited consistent pH values ranging from 6.1 to 6.5. In contrast, the wet-fractionated proteins RP, WG, and PPI, exhibited varying pH values of 4.5, 6.0, and 7.9, respectively. RP, WG, and OPC raw materials showed the lowest solubility values (measured at their original pH conditions) of 1.6%, 7.3%, and 12.3%, respectively. In contrast, PPI, and RPC exhibited intermediate solubility values of 25.6% and 36.4%, respectively, while PPC exhibited clearly higher solubility value of 64.8%. Low solubility can be explained by the isoelectric point, protein type

and processing history (Foegeding and Davis, 2011; Osborne, 1897; Pelgrom et al., 2015). For instance, commercial wet extracted raw materials are often denatured (Nisov et al., 2020b; Osen et al., 2014) due to their harsh processing conditions, while the dry fractionated proteins often remain in their native state (Pelgrom et al., 2015). The denatured proteins (REPI, WG, an PPI) tend to exhibit lower solubility, but also their ratios of protein type (Osborne classification) affect their solubility (whether they are soluble in water or in dilute alkaline conditions etc.). Moreover, the pH of both REPI and WG raw materials aligned with their isoelectric points (Kong et al., 2007; Souza et al., 2017), which makes the net protein charge zero, leading to the lowest possible solubility value for that particular protein.

REPI and WG exhibited conductivity values of 0.4 and 0.3 mS/cm, respectively, while PPI, PPC, and RPC exhibited higher values of 1.5, 1.9, and 2.5 mS/cm, respectively. The higher conductivity values of PPI, PPC, and RPC may be related to their notably higher ash content, as conductivity is directly related to the concentration of ions in a solution (Visconti et al., 2010). PPI distinguished itself with a high water-holding capacity of 4.9 g/g in comparison to RP, WG, and PPC, which exhibited values of 2.4, 1.7, and 2.5 g/g, respectively. Wet-fractionated raw materials exhibited clearly larger particle size values in comparison to those obtained through dry fractionation. PPI exhibited the highest Dx (90) and Dx (50) particle size values of 320 and 112 μm, respectively, followed by WG (191 and 94 μm) and REPI (158 and 84μm). It is worth noting that the particle size analysis was conducted in water, in order to mimic the conditions during functionality testing. Given the high water-holding capacity of PPI, it is plausible that the particles expanded during measurement, leading to the observation of larger particles. In contrast, dry-fractionated raw materials showcased notably smaller Dx (90) and Dx (50) values, ranging from 38 to 73 μm and 7.4 to 30 μm, respectively. These results can be explained by the processing conditions where the wet-fractionated ones are suspended to water and subjected to either washing at its native pH or to pH-shifting under alkaline and acidic environments, followed by a subsequent drying step (Hernández-Álvarez et al., 2023; Ortolan and Steel, 2017). These processing conditions may induce protein denaturation, particularly at industrial scale, exposing their hydrophobic groups and resulting in formation of larger protein aggregates (Lie-Piang et al., 2023). In contrast, dry fractionation involves only defatting, dry milling, and in this particular case, air classification, thereby retaining the proteins in their native state and preventing the protein aggregation into larger particles.

Table 16. Physicochemical properties of Rice endosperm protein, wheat gluten, pea protein isolate, pea protein concentrate, oat protein concentrate, and rapeseed protein concentrate.

Raw material	pH	Cond. (mS/cm)	Solubility (%)	WHC (g/g)	Particle size (μm)			Publication
					Dx (10)	Dx (50)	Dx (90)	
REPI	4.5	0.4	1.6	2.4	39	84	158	I, II
WG	6.0	0.3	7.3	1.7	42	94	191	II
PPI	7.9	1.5	25.6	4.9	34	112	320	II
PPC	6.5	1.9	64.8	2.5	4.7	15	38	II
OPC	6.4	na	8.2*	na	1.9*	7.4*	44*	III
RPC	6.1	2.5*	36.4	na	6.4	30	73	IV

*Protein content measured using Kjeldal method.

Abbreviations: REPI = rice endosperm protein, WG = wheat gluten, PPI = pea protein isolate, PPC = pea protein concentrate, oat protein concentrate, RPC = rapeseed protein concentrate, Cond. = Conductivity, WHC = water-holding capacity. Particle size of WG is measured in dry form while others in aqueous environment

The defined raw material characteristics highlighted the limited applicability of REPI in solubility-dependent food applications. Consequently, the first part (I) of this study was directed towards functionalising the insoluble REPI into smaller protein molecules to enhance its solubility. The second part of this study, involving all raw materials, (I, II, III, IV) focused on meat analogue applications that typically benefit from less soluble ingredients (Geerts et al., 2018) and high protein content (Section 2.3).

5.2 Limited enzymatic protein hydrolysis for improved functionality in solubility-dependent food applications

Protein solubility is a prerequisite for various functional properties of food proteins, encompassing emulsification, gelation, foaming, and more (Day, 2013). One approach to increase plant protein solubility involves reducing its molecular weight using limited enzymatic hydrolysis, which preserves its ability to form networks with adjacent proteins. While the effect of limited hydrolysis on emulsifying properties has been widely studied, the effects on foaming properties, colloidal stability, and gelation are scarce, particularly when considering rice proteins. In this current study, limited hydrolysis was applied on the denatured and insoluble REPI (I) with the aim of improving functional properties reliant on high protein solubility, excluding emulsifying properties. These functional properties included colloidal stability, foaming, and gelation. This process involved the use of two different enzymes, neutral and acid endoproteases. Each enzyme was employed under its optimal pH conditions, with neutral endoprotease operating at pH 7 and the acid endoprotease at pH 5. As the enzymes required different pH conditions, the resulted properties were analysed under both pH conditions. Table 17 presents the resulted physicochemical and functional properties under both pH conditions, considering three different degrees of hydrolysis for each enzyme individually.

Table 17. Degree of hydrolysis and its effect on physicochemical and functional properties of rice endosperm protein isolate (REPI).

Sample	DH (%)	Solubility (%)		Zeta potential (mV)		Surface hydrophobicity		WHC (g/g)	OHC (g/g)
		pH 5	pH 7	pH 5	pH 7	pH 5	pH 7	pH 7	pH 7
Control 0h	0.0 ± 0.0	1 ± 0	3 ± 0	-1 ± 0	-18 ± 1	48 ± 5	80 ± 13	na	na
Control 3h	0.1 ± 0.2	1 ± 0	4 ± 0	-2 ± 0	-16 ± 1	47 ± 5	118 ± 22	2.8 ± 0.1	1.0 ± 0.0
NEP 0.2U	0.2 ± 0.0	6 ± 1	9 ± 0	-5 ± 1	-21 ± 1	22 ± 1	40 ± 2	na	na
NEP 2U	1.5 ± 0.0	18 ± 0	21 ± 0	-6 ± 1	-20 ± 1	10 ± 1	15 ± 1	3.4 ± 0.1	3.1 ± 0.1
NEP 20U	4.8 ± 0.3	43 ± 0	46 ± 2	-5 ± 1	-18 ± 1	7 ± 0.4	6 ± 1	3.3 ± 0.0	3.4 ± 0.0
AEP 0.2U	0.4 ± 0.5	11 ± 0	14 ± 0	-2 ± 0	-15 ± 0	12 ± 2	12 ± 2	na	na
AEP 2U	1.9 ± 0.1	24 ± 1	28 ± 0	-1 ± 0	-11 ± 0	7 ± 1	6 ± 3	na	na
AEP 20U	5.4 ± 0.2	51 ± 0	55 ± 1	-1 ± 0	-10 ± 1	4 ± 0	3 ± 1	na	na
Sample	DH (%)	Foam capacity (%)		Foam stability (%)		Colloidal stability (%)		Gel formation	
		pH 5	pH 7	pH 5	pH 7	pH 5	pH 7	pH 5	pH 7
Control 3h	0.1 ± 0.2	35 ± 5	250 ± 0	0 ± 0	76 ± 4	2.0 ± 3	5.0 ± 8	yes	no
NEP 0.2U	0.2 ± 0.0	253 ± 15	315 ± 5	0 ± 0	84 ± 1	32 ± 0	31 ± 4	na	na
NEP 2U	1.5 ± 0.0	260 ± 3	365 ± 5	0 ± 0	85 ± 2	72 ± 1	68 ± 2	yes	no
NEP 20U	4.8 ± 0.3	230 ± 20	333 ± 3	0 ± 0	5.0 ± 1	87 ± 2	89 ± 1	no	no
AEP 0.2U	0.4 ± 0.5	415 ± 15	420 ± 0	0 ± 0	89 ± 0	16 ± 3	20 ± 2	na	na
AEP 2U	1.9 ± 0.1	497 ± 15	487 ± 0	0 ± 0	90 ± 0	20 ± 2	32 ± 2	no	no
AEP 20U	5.4 ± 0.2	413 ± 10	453 ± 7	0 ± 0	90 ± 1	86 ± 2	75 ± 1	no	no

Abbreviations: U/g = enzyme units per g protein, DH = degree of hydrolysis, WHC = water-holding capacity, OHC = oil holding capacity, NEP = Neutral endoprotease, AEP = acid endoprotease, na = not analysed.

The degree of hydrolysis was analysed for monitoring the extent of proteolysis, and solubility to observe the enzyme efficiency. It measures the changes in free amino ends in relation to the total amount of peptide bonds in a protein sample. Neutral endoprotease resulted in 0.2, 1.5 and 4.8% degrees of hydrolysis while acid endoprotease exhibited slightly higher values of 0.4, 1.9 and 5.4%. The limited hydrolysis (0.2-5.4%) resulted in a notable improvement in solubility values, from 1% to 6-51% at pH 5 and from 3% to 9-55% at pH 7. Similarly, Paraman et al. (2007) and Xu et al. (2016) reported an increase in solubility as a function of elevated degree of hydrolysis. In their studies, rice endosperm protein and rice glutelin exhibited an increase in solubility, increasing from 12% to 19-26% and from 5% to 78-85%, respectively. These shifts corresponded to degree of hydrolysis values of 5.2-13.5% for rice endosperm protein and 0.5-6% for rice glutelin.

Zeta potential was measured as it indicates the electrostatic repulsion between particles, providing insights about the resulted changes in colloidal stability. Although the protein solubility remained comparable under both pH conditions, zeta potential values exhibited a notable difference not only in terms of pH but also regarding enzyme. Acid endoprotease resulted in zeta potential values of 1-2 mV at pH 5 and 10-15 mV at pH 7, while neutral endoprotease led to values of 5-6 mV at pH 5 and 18-21 mV at pH 7. The highest values were observed at the lowest degrees of hydrolysis for both enzymes. However, the values did not reach the threshold of ±30 mV for considering a suspension as stable. Zheng et al. (2020), who studied oat protein hydrolysates with degrees of hydrolysis ranging from 2% to 16%, reported zeta potential values nearing zero (-3 to -1 mV), with the exception of an almost -8 mV value at an intermediate degree of hydrolysis of 6%. This was in contrast to the current study, which exhibited the highest values at the lowest degrees of hydrolysis (0.2-0.4%) with both enzymes.

Surface hydrophobicity was measured to understand how proteolysis changes the behaviour of the protein in an aqueous suspension and the subsequent effect on their colloidal stability, foaming and gelation properties. Surface hydrophobicity of REPI increased from 72 to 118 after the control treatment at pH 7 but remained at 48 at pH 5. Both enzymes induced a decline in these values with increased hydrolysis. Acid endoprotease reduced surface hydrophobicity more (3-12) than neutral endoprotease (6-40). The latter showed more variation, especially at lower hydrolysis degrees, with hydrophobicity values of 15-40 at pH 7 and 10-22 at pH 5 for 0.2-1.5% hydrolysis. Paraman et al. (2007) also observed a decreasing trend in surface hydrophobicity values as the degree of hydrolysis of rice endosperm protein increased, regardless of the enzyme and optimal emulsification properties at intermediate degrees of hydrolysis. In contrast, Xu et al. (2016) reported that the highest surface hydrophobicity value for rice glutelin hydrolysate occurred at a degree of hydrolysis of 2%, which also corresponded to the optimal emulsification conditions.

Colloidal stability increased with an increasing proteolysis and was improved from 2-5% to 87-89%. Interestingly, colloidal stability values were minimally affected by the pH conditions, although the zeta potential values would suggest otherwise, as the dispersion stability often correlates with high zeta potential values (Fang et al., 2020; Liu et al., 2011; Nivala et al., 2017). On the other hand, the decreased surface hydrophobicity suggested that the hydrophilic interactions increased, which could explain the higher solubility and colloidal stability. To the best of available knowledge, other studies investigating the limited degree of hydrolysis did not report its effect on colloidal stability.

Limited hydrolysis of REPI by neutral endoprotease had a positive impact on both water and oil-holding capacities at the studied degrees of hydrolysis of 1.5% and 4.8% at pH 7. Vioque et al. (2000) studied the effect of limited hydrolysis of rapeseed proteins on water and oil-holding capacities. In their study, oil and water-holding capacities increased at degree of hydrolysis of 3.1%, after which the values started to decline. In fact, they concluded that above a certain degree of hydrolysis, the gained benefit was lost due to a smaller size of the resulted peptides.

The increased water-holding capacity observed in the pH 5 control (3 h) treatment and the treatment leading to a 1.5% degree of hydrolysis at pH 5 correlated with the gelation results, as these treatments, using no enzyme or neutral endoprotease, were the only ones that induced gelation. However, this observation alone does not fully explain the improved gelation, as the water-holding capacity was similar at higher degrees of hydrolysis and at 1.5% degree of hydrolysis at pH 7. The clearest increase was observed between the pH 7 and 5 controls, suggesting that the increased gelation was more closely related to the lower pH than to the hydrolysis treatment. However, using acid endoprotease did lead to destruction of gel structure even at pH 5. Nieto-Nieto et al. (2014) reported increased gel hardness and water-holding capacity at pH 9 for oat protein hydrolysates produced using a wide range of enzymes. Interestingly, hydrolysate that was produced by pepsin, failed to form a gel despite achieving a similar degree of hydrolysis to other samples. This particular hydrolysate was digested by pepsin at pH 2, while other enzymes required higher pH conditions ranging from pH 7 to 8. Nieto-Nieto et al. (2014) suggested that the acidic hydrolysis environment resulted in partially unfolded oat proteins, making them more susceptible to digestion by pepsin. In this study, the acid endoprotease required treatment at pH 5, presumably inducing similar effect on rice proteins, making them unsuitable for gelation.

Unhydrolysed REPI at pH 5 exhibited poor foaming capacity (35%) and zero foam stability. These conditions were in proximity to its isoelectric point, where the net charge of the proteins becomes zero. Significant improvement in foaming properties was achieved already by shifting the pH away from the isoelectric point, specifically to pH 7. This shift reached foam capacity and stability values of 250% and 76 % respectively. Although the foam stability increased from zero to 76%, the foam quality remained poor, characterised by the presence of air cavities. As the unmodified REPI at pH 7 exhibited lower water-holding capacity (2.8 g/g) and high surface hydrophobicity, these air cavities allowed the water to drain from the solids, resulting in a dry foam network (Figure 6). Although the foam capacities in this study were improved by limited hydrolysis at pH 5, the foam stability remained zero and exhibited exceptionally poor quality (Figure 6). The greatest improvements in foam capacity and stability values were observed after hydrolysis by the acid endoprotease at pH 7 corresponding to values 487% and 90%, respectively, with 1.9% degree of hydrolysis. However, similarly to the control foams, the foam quality was dry and exhibited air cavities (Figure 6B). The only treatment that resulted in foam quality without air cavities even after 10 min of standing was achieved using neutral endoprotease with 1.5% degree of hydrolysis at pH 7 (Figure 6A). This treatment corresponded to foam capacity and stability values of 365% and 85 %, respectively. These results did not only emphasise the importance of limited hydrolysis but also the selection of an enzyme that would favour the properties required in certain functional properties.

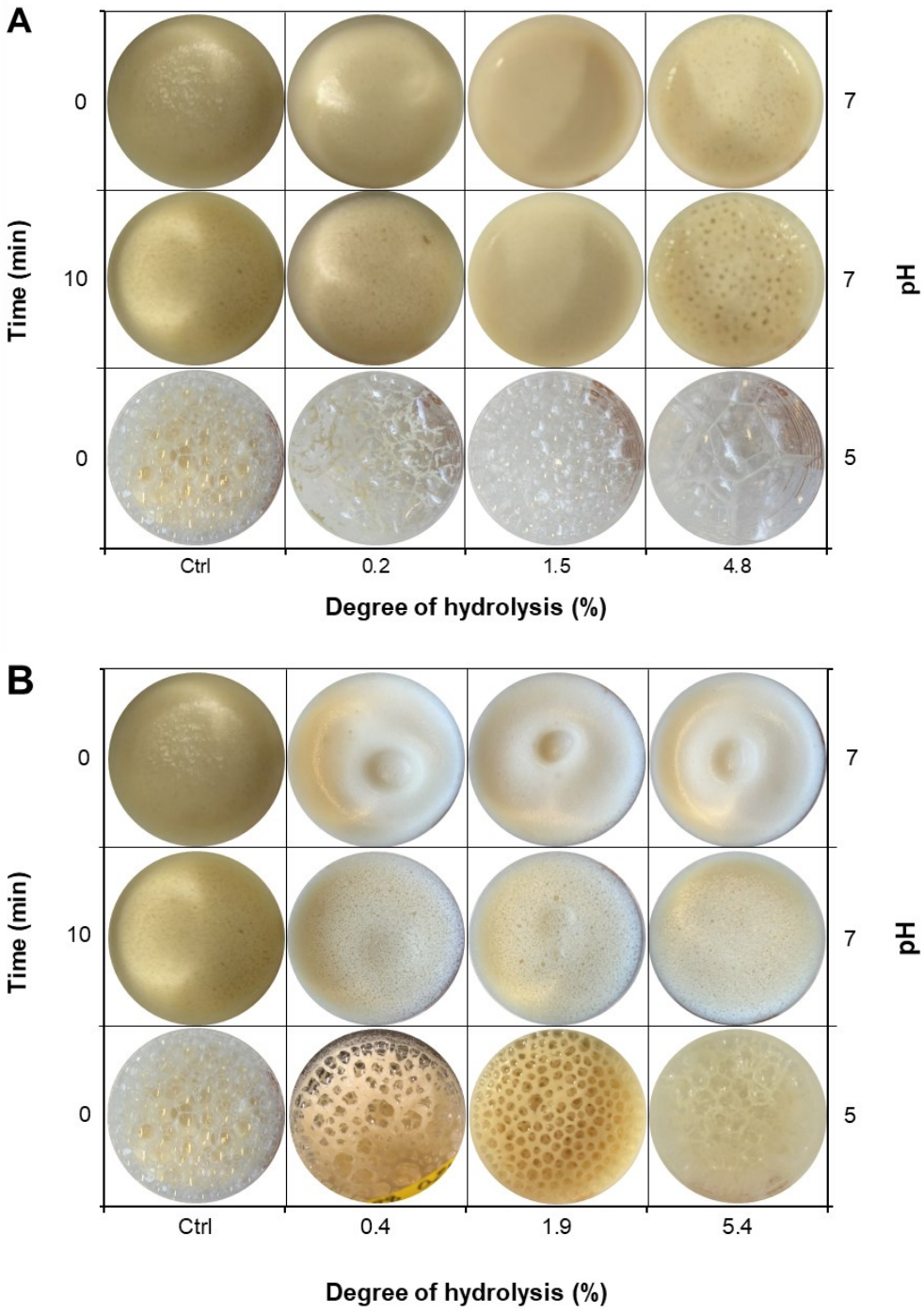


Figure 6. Visual illustration of the effect of limited hydrolysis on foam quality of rice endosperm protein at various degrees of hydrolysis using A) neutral and B) acid endoproteases under pH 5 and 7. Foam quality after specific period of standing is indicated by time (min).

In line with the current study, Vioque et al. (2000) demonstrated a threefold foam capacity in rapeseed protein isolate after protease treatment. Specifically, they reported foam

capacity of 69% at 3.1% degree of hydrolysis corresponding to 301% increase in foam volume – a threefold improvement compared to the unhydrolysed rapeseed protein isolate. Additionally, this treatment induced a notable increase in water-holding capacity, reaching 5.9 g/g. Further hydrolysis of rapeseed protein exhibited a decreasing trend in both foaming and water-holding capacities, consistent with the findings from this study related to foaming.

The unmodified REPI showed three major bands at 13, 20, and 32 kDa, presumably identified as the predominant prolamin (or globulin), β-glutelin, and α-glutelin subunits, respectively (Figure 7). Additionally, less prominent bands were detected at 11 (globulin), 16-17 (prolamin), 26 (globulin), and 54 (globulin) kDa. The 13 kDa band remained unchanged regardless of the degree of hydrolysis and enzyme used, indicating that it was not a suitable substrate for the studied enzymes. On the contrary, both enzymes were effective on the glutelin subunits, which showed fainted bands at 4.8 and 5.3 % degree of hydrolysis. After 5.3 % proteolysis only the 13 kDa bands and under remained, which suggested already quite drastic proteolysis. Hydrolysis reduced the intensity of the proteins retained in the loading well, suggesting an effective decrease in the protein aggregation. The REPI supernatant lanes exhibited peptide bands under 25 kDa that gradually decreased with the degree of hydrolysis. This was in accordance with the study by Xu et al. (2016), that demonstrated gradual decrease in molecular weight of rice glutelin when hydrolysed by trypsin. Although the peptide size and degree of hydrolysis (0.5-6%) was similar to the current study, they reported tenfold higher values for surface hydrophobicity (350-450).

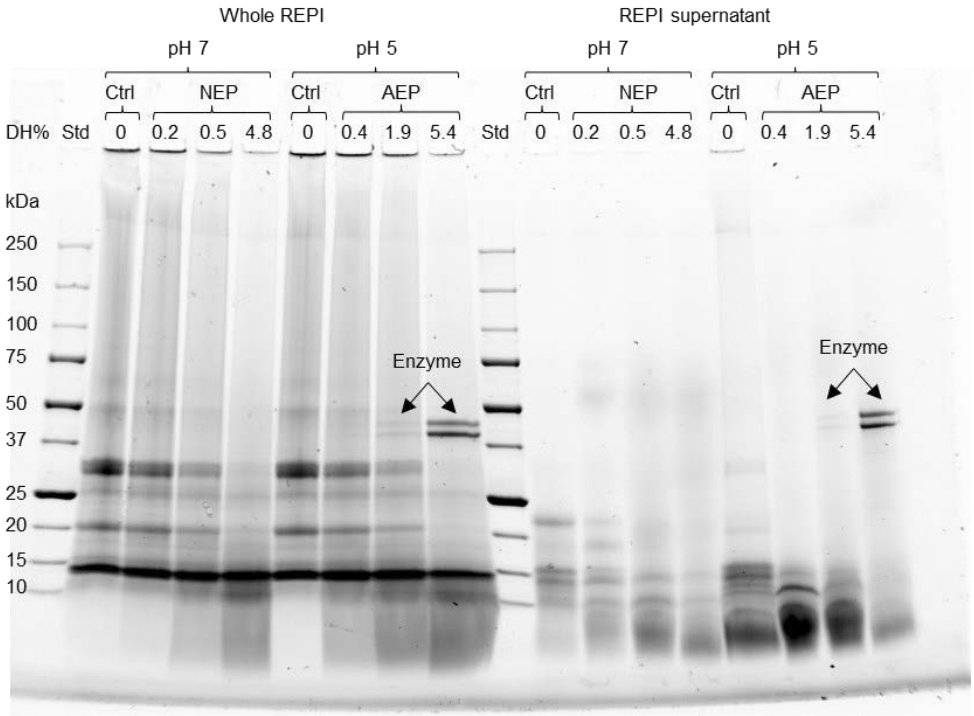


Figure 7. Protein and peptide profiles of rice endosperm protein (REPI) and its hydrolysates at different degrees of hydrolysis (DH), after treatments with neutral (NEP) and acid (AEP) endoproteases under pH 7 and 5, respectively. Ctrl stands for control sample without added enzyme.

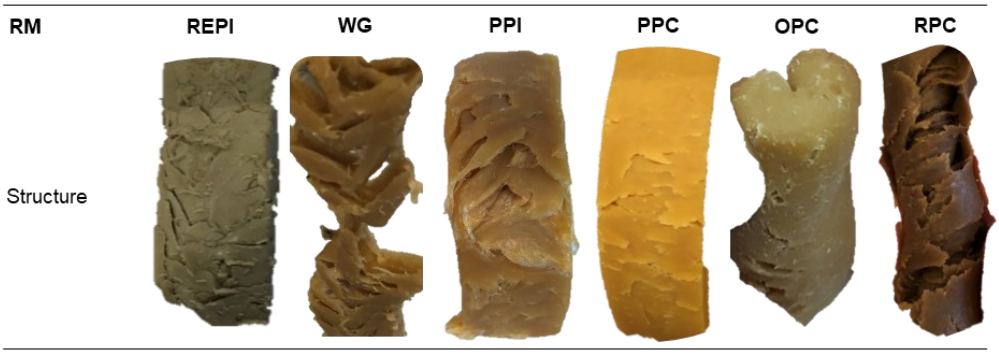
In conclusion, limited hydrolysis is a promising tool for improving functional properties of originally insoluble proteins derived from rice. The resulted outcome varied according to the studied functional property. For example, the optimal degree of hydrolysis was different for foaming properties compared to colloidal stability. Nevertheless, the intermediate limited hydrolysis was generally preferable, particularly when improving foaming properties. Furthermore, the selection of enzyme played a major role in the resulted functionality, as different enzymes yielded diverse outcomes, even when the degree of hydrolysis was similar.

5.3 Functionalisation strategies for improved performance in meat analogue application

The second part of this study further elaborated the applicability of REPI proteins in creation of fibrous structures for meat analogue applications. Moreover, a primary focus was the identification of effective functionalisation strategies to enhance fibrous structure formation of plant-based ingredients with low protein content (PPC, OPC, and RPC). To strengthen the understanding of structure formation during HMEP, two well-established protein isolates, namely PPI and WG, were studied alongside the other protein ingredients (REPI, PPC, OPC, RPC). While many other food products such as puddings, meringue, and milk rely on highly soluble proteins, meat analogues distinguish themselves as products requiring less soluble proteins (Geerts et al., 2018; Osen et al., 2014).

Figure 8 illustrates the optimal structures of the investigated raw materials that could be achieved prior to any functionalisation in HMEP. It is worth mentioning that all the studied temperatures during HMEP are expressed as the material temperature, referred to as melt temperature, inside the extruder die body, right before entering to the cooling die. WG and PPI demonstrated robust fibrous structures already in their unmodified state at 146 °C and 122 °C, respectively, aligning with the extensive research previously conducted on wheat and pea proteins (see Section 2.3). On the contrary, research on rice proteins during HMEP is scarce and has not yet demonstrated the formation of robust fibrous structures (Zhang et al., 2023). Interestingly, the commercial insoluble REPI investigated in this study revealed relatively strong structure formation ability already at its unmodified state at 156 °C. Among protein concentrates, particularly OPC with its protein content of 39.5%, no fibrous structures were observed in its unmodified state. Instead, it formed dough-like, stretchy structures across a wide range of temperatures (90-160 °C). On the other hand, PPC with higher protein content of 48.5%, started to demonstrate a slightly more rigid structure at melt temperature of 141 °C without the stretchiness but it failed to demonstrate robust fibrousness even at elevated temperatures. In contrast, RPC exhibited an acceptable layered structure despite its low protein content of 41.1%. However, the presence of other components, such as 26.7% dietary fibre (IV), presumably influenced the overall structure of RPC, making it somewhat soft. To the best of available knowledge, no previous research has been published on pea and oat protein concentrates as a single ingredient for fibrous structure formation. Regarding rapeseed, one prior research has been conducted using a 56% rapeseed protein concentrate in shear cell technology, yielding similar results to the current HMEP study, demonstrating evident fibrous structure formation at temperatures of 140 °C and above (Jia et al., 2021a).

Figure 8 also highlights the varying melt temperatures required for optimal structure formation of the unmodified materials. For instance, REPI required a high melt temperature of 156 °C, while PPI showed robust protein alignment already at 122 °C. These variations could be attributed to their different cysteine contents, which is the only amino acid possessing a thiol group, thereby enabling it to form disulphide bonds. Additionally, the proportion of free thiol groups ready for covalent disulphide crosslinking may play a crucial role, given that disulphide bonding has been identified as the main protein-protein interaction required for stabilising fibrous structures (Liu and Hsieh, 2008; Osen et al., 2014; Zhang et al., 2022b). In thermomechanical processing the protein starts to uncoil and rearrange as the thiol groups and disulfide bonds become more reactive and labile at elevated temperatures (Cornet et al., 2022).



RM	REPI	WG	PPI	PPC	OPC	RPC
Structure						
pH	4.5	6.0	7.9	6.5	6.1	6.4
Protein (%)	72.0	73.1	74.4	48.5	39.5	41.1
Solubility (%)	1.6	7.3	25.6	64.8	8.1	36.4
Melt °C	156	142	122	141	152**	142
Cysteine (%)	1.6	1.8	0.9	1.2	na	na
Thiols (µmol/g)	0.8*	0.9*	0.5*	7.6*	3.8	27.2

Figure 8. Structure formation of unmodified rice protein (RP) wheat gluten (WG), pea protein isolate (PPI), pea protein concentrate (PPC), oat protein concentrate, and rapeseed protein concentrate (RPC) at material-specific pH conditions and melt °C as well as their cysteine and free thiol groups concentrations. * Control samples from (II). ** Control sample (III). Thiols denote for free thiol groups present in each unmodified ingredient.

Among the protein concentrates, RPC showed a notably higher amount of free thiol groups of 27 µmol/g, along with better structure formation ability compared to other protein concentrates that exhibited 3.8-7.6 µmol/g. Interestingly, the lowest free thiol group level was measured in OPC, corresponding to its non-existing fibrous structure, while the intermediate value was measured in PPC, aligning with the slightly more rigid structure. Among protein isolates, the free thiol groups were considerably lower, ranging from 0.5-0.9 µmol/g, which can be associated with their production through wet extraction that tends to aggregate the proteins, likely resulting in the lower amount of detected free thiol groups. However, HMEP is able to uncoil this aggregated structure and enables rearrangement of disulphide interactions. For a more comprehensive comparison, this aspect would require exploring the total amount of thiol groups present, as well as the sulphur-containing cysteine contents in all of these raw materials. This is particularly relevant as PPI, despite its pronounced multilayered structure, exhibited the lowest levels of cysteine and free thiol groups. Moreover, it has been reported that the conversion of intramolecular disulphide bonds into intermolecular disulfide bonds during HMEP is particularly cru-

cial for fibrous structure formation (Zhang et al., 2022b). Furthermore, the proportion of these intermolecular bonds were significantly lower than that of intramolecular interactions. Given this observation, the total number of thiol groups may not be the most significant factor, but the distribution of these groups within the protein molecules and their availability to reach to adjacent protein molecules.

5.3.1 Effect of pH on raw material characteristics and fibrous structure formation

As a continuation to the limited hydrolysis study, we applied the optimum functionalisation conditions (1.5% hydrolysis at pH 7) in HMEP. Preliminary findings showed improved fibrous structures at lower temperature compared to the unmodified REPI, but this was accompanied with process instability and excess water leakage demonstrating the unsuitability of increased solubility in HMEP for this particular ingredient. It was concluded that only the pH shift from the original acidic conditions to neutral was sufficient to improve the structure. Therefore, to delve deeper into the relationship between pH and fibrous structure formation, the second study (II) investigated REPI, PPC, PPI, and WG at three different extrusion temperatures under pH conditions of 5 and 7. It is worth noting that preliminary trials, which included control treatment involving only freeze-drying without pH-shift, showed no change in fibrous structure formation. Therefore, this study concentrated on wider range of raw materials rather than including numerous pH points and control treatments.

Extrudate characteristics

The studied melt temperatures were selected based on the ability of each raw material to form fibrous structures in HMEP characterised by three distinct degrees of fibrousness. The first degree, representing a lack of structure, was observed under extrusion conditions below 95 °C, regardless of the raw material and pH conditions. This aligns with similar findings reported by Pietsch et al. (2017) in gluten at 110 °C and by Osen et al. (2014) in PPI at 100 °C, where unorganised structures were observed under these lower extrusion temperatures. The second and third degrees, corresponding to the lowest achievable fibrillation temperatures at pH 7 and 5 respectively, varied depending on the raw material. REPI, WG, and PPC began to form fibrous structures at melt temperatures of 140 °C and 160 °C. In contrast, PPI started to form fibrous structures at lower melt temperatures of 115 °C and 135 °C. This observation is consistent with previous findings that identified the temperature threshold for fibrous structure formation in PPI as 120 °C during HMEP (Osen et al., 2014).

Figure 9 shows the macrostructures and microstructures of the resulted extrudates at the two highest temperatures for each raw material under pH 5 and 7. One of the major findings was that the pH shift to neutral conditions facilitated the formation of fibrous structure at lower melt temperatures for all raw materials regardless of their isoelectric points. While microstructures of the extrudates at pH 5 at the lower temperatures corresponded to unorganised clusters lacking clear protein alignment, at elevated temperatures and pH conditions the protein alignment became detectable. Particularly noteworthy improvement was the pronounced structural alignment observed in the low-protein PPC sample under the highest studied temperature and pH conditions (160 °C, pH 7), showcasing a protein network seen as thin and evenly distributed “lines”.

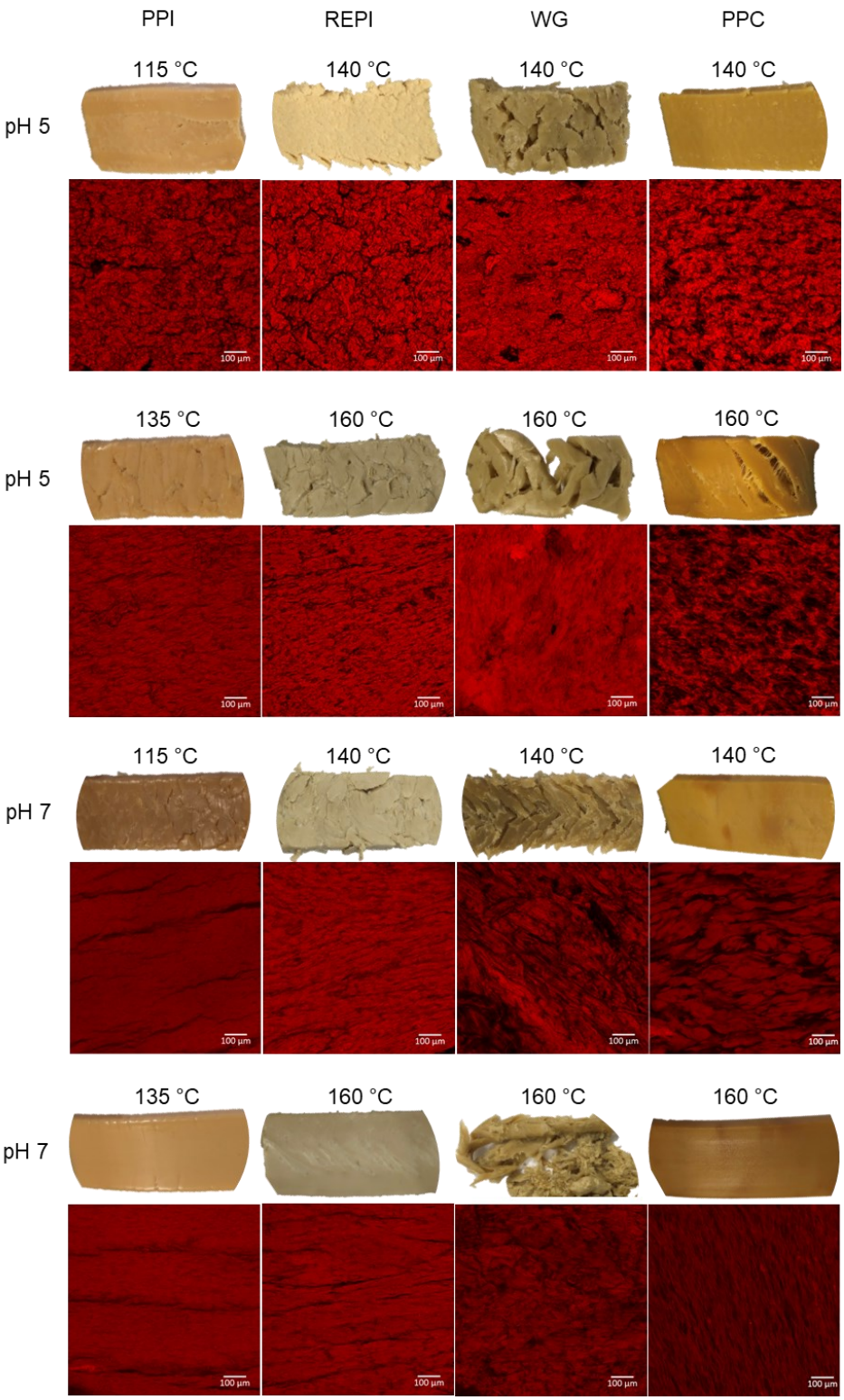


Figure 9. Effect of pH and temperature on macrostructure and microstructure of pea protein isolate (PPI), rice protein (RP), wheat gluten (WG), and pea protein concentrate (PPC), with protein staining using acid fuchsin seen as red.

Despite the low protein content of PPC (48.5%), this structure resembled the microstructures reported by Chiang et al. (2019) for wheat and soy protein blends, as well as for cooked chicken breast. In addition, a unique macrostructure was observed for WG at pH 7 (160 °C), which emerged from the extruder as elongated and narrow fibres unlike the other samples that retained the shape of the cooling die. However, it is important to note that the processing of WG pH 7 samples was somewhat instable.

Shifting the pH from 5 to 7 not only resulted in more pronounced alignment but also produced stronger extrudates, as evidenced by tensile strength measurements depicted in Table 18. The tensile strength values increased in both longitudinal and perpendicular directions. Generally, all extrudates demonstrated increased tensile strength values also at elevated temperatures, consistent with the micro and macrostructures. This aligns with previous studies on wheat, soy and rapeseed proteins that showed an increased tensile stress as a function of increasing temperature and tensile stress (Jia et al., 2021a). Interestingly, the PPC sample corresponding to melt temperature of 160 °C demonstrated comparable tensile strength values to PPI at 135 °C under pH 7 conditions, which suggested that the pH shift to 7 was an effective tool to functionalise protein concentrates for enhanced fibrous structure formation during HMEP.

Table 18. Effect of pH and temperature on tensile strength and free thiol groups of rice protein, wheat gluten, pea protein concentrate, and pea protein isolate raw materials. The statistical significance ($p < 0.05$) is denoted by the superscript letters within a group. Each column of the table corresponds to one group.

Sample	Tensile strength (kPa)				Free thiol groups (µmol/g)			
	P	L	P	L	RM	Extrudates		
	140 °C		160 °C		Control	95 °C	140 °C	160 °C
REPI pH 5	23	12	129	66	0.8^{d}	0.6^{d}	0.9^{d}	2.5^{b}
REPI pH 7	161	169	276	212	0.7^{d}	0.5^{d}	1.5^{c}	3.5^{a}
WG pH 5	7.8	3.1	14	5.4	0.9^{c}	0.6^{e}	1.5^{b}	1.9^{a}
WG pH 7	8.9	13	8.8	12	0.9^{c}	0.7^{de}	0.9^{cd}	1.1^{c}
PPC pH 5	54	19	115	45	7.9^{ab}	7.7^{b}	4.8^{d}	4.7^{d}
PPC pH 7	101	66	140	128	7.6^{b}	8.3^{a}	5.9^{c}	6.0^{c}
	115 °C		135 °C		Control	95 °C	115 °C	135 °C
PPI pH 5	17	14	90	16	0.5^{d}	1.3^{ab}	0.7^{c}	0.9^{c}
PPI pH 7	29	13	76	150	0.5^{d}	1.3^{b}	0.9^{c}	1.5^{a}

Abbreviations: REPI = rice endosperm protein, WG = wheat gluten, PPC = pea protein concentrate, PPI = pea protein isolate, RM = raw materials, P = perpendicular, L = longitudinal

Research investigating the effect of pH on fibrous structure formation during HMEP at varying temperatures, remains scarce. One of the earliest studies regarding HMEP using cooling die, reported an increased tensile strength for soy protein isolate at 180 °C, when pH was shifted from acidic condition to neutral, which was in accordance with the current study (Noguchi, 1989). A more recent study by Muhialdin & Ubbink, (2023) investigated extruded PPI matrices under 50% moisture content using a short circular die. They examined varying pH conditions ranging from 5 to 9 reaching only extrusion temperatures of 105 °C, insufficient for generating fibrous structure in PPI (as demonstrated above). Although the results are not directly comparable to the current study, it is worth noting that they evidenced an almost linear decrease in hardness values of the extrudates as a function of pH. This would suggest an extrudate strength trend opposite to this study. However, it is unclear how the hardness value in their study compares to the tensile strength measurements in this study, especially as their samples did not reach the temperature required for fibrous structure formation. A more recent study by Ellwanger et al. (2024), investigated the effect of pH on fibrous structure formation in soy protein isolate (at pH 4.1-7.4) and

wheat gluten (pH 3.4-5.8) during HMEP using a long cooling die. Their findings aligned with the current study, demonstrating a gradual decrease in fibrous structure formation, as the pH declined.

Regarding thiol group interactions, the dry fractionated PPC showed higher amount of free thiol groups compared to the wet-extracted REPI, WG, and PPC as explained in the beginning of the section 4.3. The number of free thiol groups in all REPI, WG, and PPC extrudates produced at 95 °C decreased (or remained the same) compared to the raw materials with the exception of PPC showing an increase from 7.6 to 8.3 µmol/g at pH 7. PPI exhibited an opposite trend showing decreased free thiol groups compared to the raw material. Zhang et al. (2022b) demonstrated that during a successful fibrous structure formation reaction in HMEP, the intermolecular disulphide bond formation gradually increases from the mixing zone to extrudate, although the ratio in relation to intramolecular disulphide bonding is small. Additionally, they observed that certain types of intramolecular disulphide bonds increase while others can decrease. In the current study, it is not possible to distinguish intra- and intermolecular disulphides from each other. However, as no fibrous structure was observed in extrudates produced at 95 °C, it suggests that the proteins were preferably forming intramolecular disulphide bonds instead of intermolecular bonds making the protein molecules tighter.

With an increase in the temperature, the free thiol groups of REPI, WG, and PPC extrudates increased. Again, PPI displayed a unique trend, initially showing a decreased trend at 115 °C and then an increasing trend towards higher temperature. This different trend could be attributed to the lower temperatures required for PPI to form fibrous structures. A similar trend in free thiol groups, specific to PPI, was observed by Sun et al. (2022) at HMEP temperatures of 90 °C, 120 °C, and 130 °C.

During the thermomechanical processing of proteins, the reactivity of free thiol groups increases at elevated temperatures (Cornet et al., 2022). Simultaneously, existing disulphide bonds become unstable and begin to engage in thiol-disulphide interchange reactions. The samples in the current study exhibited increased fibrous structures at elevated temperatures, suggesting that the original disulphide bonds broke down at these temperatures and rearranged to form intermolecular disulphide bonds with adjacent protein molecules. This forced the proteins to align into a stabilised network during the cooling phase, and simultaneously prevented the intensive intramolecular disulphide bonding observed as the increased number of free thiol groups at higher temperatures.

Regarding the pH, lower number of free thiol groups was observed at the isoelectric points of the proteins. Interestingly, regardless of this observation, the fibrous structure formation was more prominent at pH 7 in notably lower temperatures. This phenomenon can be explained by the enhanced reactivity of disulphide bonding at neutral or higher pH conditions, particularly when protein has been subjected to heating, which makes it susceptible for disulphide bond reduction and subsequent rearrangement upon cooling phase (Rajpal and Arvan, 2013; Volkin and Klibanov, 1987).

Raw material characteristics

Previous research has shown that changes in pH conditions, or pH-shifting, can greatly impact protein properties, such as protein solubility, water-holding capacity, and particle size (Kong et al., 2007; Peng et al., 2020; Tang et al., 2023). Some studies have investigated these properties in relation to fibrous structure formation (Geerts et al., 2018; Osen et al., 2014). Therefore, this study concentrated on characterising raw material properties

such as solubility, water-holding capacity, and particle size, as well as conductivity (Table 19). These attributes were analysed in the unmodified state of the proteins and after undergoing various treatments, including a control treatment without pH alteration, and pH adjustments to 5, 7, and 8. A comparison of the results between the unmodified and control states ruled out the impact of freeze-drying on the outcomes during HMEP, as similar conductivity, solubility, and water-holding capacities were observed under both conditions (Table 19). As an exception, WG exhibited significant increase in water-holding capacity after the control treatment. However, preliminary extrusion trials showed that these changes had minor influence on the outcomes during HMEP. A study by Osen et al. (2014) supported these findings, as they evidenced similar fibrous structures in three different commercial pea protein isolates, despite their varied water-holding capacities ranging from 2.1 to 5.4 ml/g. Overall, they concluded that differences in functional properties of these raw materials played a minor role in structure formation during HMEP.

Table 19. Raw material characteristics of pH-shifted plant proteins. Different letters from a to d are designated for significant differences ($p < 0.05$) between the values in each raw material within one column.

Sample		pH	Cond. (mS/m)	Solubility (%)	WHC (g/g)	Particle size (µm) Dx (10)	Dx (50)	Dx (90)
RP	unmodified	4.5^{c}	0.4^{b}	1.6^{b}	2.4^{b}	39^{c}	84^{c}	158^{b}
pI ≈ 5	ctrl	4.5^{c}	0.4^{b}	1.5^{b}	2.5^{ab}	46^{b}	88^{ab}	163^{a}
	shifted	4.9^{b}	0.5^{b}	1.5^{b}	2.6^{ab}	46^{b}	89^{a}	163^{a}
	shifted	7.0^{a}	1.2^{a}	3.1^{a}	2.7^{a}	48^{a}	87^{b}	138^{c}
WG*	unmodified	6.0^{b}	0.3^{c}	7.3^{c}	1.7^{d}	12^{d}	52^{c}	122^{d}
pI ≈ 7	ctrl	6.0^{c}	0.3^{c}	8.2^{b}	2.8^{b}	21^{b}	76^{a}	200^{a}
	shifted	4.9^{d}	0.5^{b}	58.3^{a}	4.3^{a}	23^{a}	76^{a}	170^{c}
	shifted	6.8^{a}	0.4^{a}	4.2^{d}	2.6^{c}	15^{c}	62^{b}	177^{b}
PPC	unmodified	6.5^{b}	1.9^{b}	64.8^{b}	2.5^{a}	5^{b}	15^{b}	38^{a}
pI ≈ 5	ctrl	6.5^{b}	2.0^{b}	69.4^{b}	2.8^{a}	5^{b}	17^{b}	39^{a}
	shifted	5.2^{c}	2.7^{a}	24.3^{c}	2.5^{a}	27^{a}	261^{a}	666^{b}
	shifted	7.2^{a}	2.2^{b}	88.7^{a}	2.8^{a}	5^{b}	17^{b}	38^{a}
PPI	unmodified	7.9^{a}	1.5^{c}	25.6^{a}	4.9^{a}	34^{d}	112^{b}	320^{b}
pI ≈ 5	ctrl	8.0^{a}	1.6^{bc}	22.3^{ab}	4.7^{a}	64^{b}	248^{a}	554^{a}
	shifted	5.4^{b}	2.4^{a}	4.9^{c}	3.5^{b}	50^{c}	130^{b}	300^{b}
	shifted	7.2^{a}	1.8^{b}	17.7^{b}	4.5^{a}	66^{a}	259^{a}	603^{a}

* Analysed in dry form

Abbreviations: REPI = rice protein; WG = wheat gluten, PPC = pea protein concentrate; PPI pea protein isolate, Cond. = conductivity, WHC = water-holding capacity, pI = isoelectric point.

In contrast to other measured attributes, following the control treatment, all raw materials exhibited larger particles, with the increase in PPC particle size being insignificant ($p>0.05$). This suggested that the process of mixing in water and subsequent freeze-drying resulted in the formation of agglomerates, likely due to the changes in hydrophobic groups of the proteins and their interaction with water as was evidenced in Publication I for REPI. While research exploring the connection between particle size and fibrous structure formation is limited, a study by Osen et al. (2014), along with the preliminary trials of this study, indicated that changes in particle size of the raw materials are not influencing structure formation during HMEP.

In the current study, WG raw material demonstrated different isoelectric point compared to other raw materials. REPI, PPC, and PPI all exhibited their lowest protein solubility values at pH 5 (1.5%, 24%, and 4.9%, respectively), while WG demonstrated its lowest solubility at pH 7 (4.2%). Furthermore, WG demonstrated its highest solubility value of 58% at pH 5, which is usually the isoelectric point for other cereal proteins. Conversely, RP, PPC, and PPI demonstrated their highest solubility values at pH 7, corresponding to

3.1, 88.7, and 17.7%, respectively. In summary, all raw materials exhibited increased solubility values further from their isoelectric points and appeared the lowest near the isoelectric point. These values aligned with previous studies that reported isoelectric points for rice (Paraman et al., 2008) and pea (Cui et al., 2020) proteins at pH 4-5, while for wheat proteins (Kong et al., 2007) at pH 7.

The opposite isoelectric point of WG to other raw materials, proved to be highly valuable when evaluating the effect of elevated pH on the formation of fibrous structures through disulphide bonding during HMEP. As clearly demonstrated in Table 4, solubility and water-holding capacity values positively correlated with one another, showing higher values for all raw materials further away from the isoelectric point. For example, when pH was adjusted further from the isoelectric point of WG, it exhibited a significant increase in both water-holding capacity and solubility at pH 5. Similarly, when the pH conditions of REPI, PPI, and PCC were adjusted further from their isoelectric points (pH 7), they exhibited an increase in water-holding capacity and solubility values. However, all raw materials exhibited more pronounced fibrous structures only at higher pH conditions, regardless of their isoelectric point, solubility, particle size, and water-holding capacity. Similarly, Ferawati et al. (2021), Kantanen et al. (2022), and Osen et al. (2014) reported no correlation between fibrous structures and water-holding capacity during HMEP. In contrast, Geerts et al. (2018) observed a positive correlation between the fibrous structure formation and an increased water-holding capacity, as well as intermediate solubility in pre-toasted soy protein fraction during shear cell structuring.

Generally, particle size distributions were analysed in water, aligning with conditions during HMEP. As an exception was made for WG, which was analysed in dry form, as it formed over 0.5 mm agglomerates in an aqueous environment. The laser diffraction method used for the measurement relies on light scattering from the particle itself, hence particles that solubilise in water during the measurement remain undetected. The water-holding capacity also influences the particle size when measured in. These factors particularly affected the particle size distributions of PPC and PPI samples. PPI, with its high water-holding capacity, showed large particles at pH 7, while PCC demonstrated small particles, influenced by the “invisibility” of the larger soluble protein particles at pH 7. REPI, which showed minimal differences in both solubility and water-holding capacity regardless of the pH conditions, exhibited less variation in particle size. Despite variations in particle size trends across all raw materials, the strongest structural alignment of the proteins was always observed at pH 7. Therefore, similarly to Osen et al. (2014), results in this study suggested a minimal correlation between particle size and fibrous structure formation.

To conclude, the findings indicated that the pH of the raw material during HMEP significantly influenced the fibrous structure formation. However, unlike solubility and other solubility-dependent functional properties, fibrous structure formation, was not dependent on isoelectric point of the ingredient. In contrast, the formation of enhanced fibrous structures was consistently more pronounced under neutral pH conditions compared to acidic ones. This phenomenon was attributed to the increased reactivity of disulphide bond formation under elevated pH conditions.

5.3.2 The effect of crosslinking and deamidation on fibrous structure formation

The unmodified OPC, produced through dry fractionation, was a low-protein (39.5%) raw material rich in starch (35%). It showed low protein solubility of 8.1% in water (12.3 % when using the Lowry method), small particle size, and poor fibrous structure formation ability (Figure 8). Therefore, this study aimed to improve the fibrous structure formation of OPC through enzymatic crosslinking and deamidation reactions, coupled with pre-heating (III). Two different dosages, including 0.5, and 5.0 U/g OPC, were studied with both enzymes, and are hereafter referred to as lower and higher dosages, respectively. Additionally, a starch degrading enzyme, α-amylase, was employed for understanding the role of starch during these treatments and in HMEP.

Table 20 summarises the pH, solubility, particle size and viscosity after various crosslinking and deamidation treatments of OPC. The control and transglutaminase treatments resulted in solubility values similar to the native OPC, ranging between 9.6-15%. Conversely, the combined trans- and protein-glutaminase treatments increased the solubility to 26% with the lower dosage and to 85% using the higher dosage. However, when pre-heating was applied, the solubility decreased to 76%, while the enzymatic starch degradation showed little effect on protein solubility. Previous studies, also those on oat proteins, have demonstrated a notable increase in protein solubility after deamidation, while the solubility tended to remain the same or slightly lower after transglutaminase treatment (Jiang et al., 2015; Liu et al., 2011; Nivala et al., 2017; Siu et al., 2002).

Table 20. Effect of pre-heating, enzymatic crosslinking, deamidation, and starch degradation (using α-amylase) on physicochemical properties of oat protein concentrate.

Sample	t	pH	Protein solubility (%)		Particle size (μm)			Viscosity*
	(h)		Lowry	Kjeldahl	D10	D50	D90	(cP)
OPC Native	0	6.3	12.3	8.2	1.9	6.6	49.3	695
Control	4	6.3	15.4	11.0	2.7	25.9	85.4	630
H-control	4	6.5	11.7	6.5	17.2	69.7	172.0	539
TPG 0.5U	4	6.5	25.5	na	2.0	5.6	25.8	501
TPG 5U	4	6.6	84.9	79.4	1.9	4.0	8.0	430
H-TPG 5U	4	6.8	76.0	59.3	10.8	29.3	69.5	1073
TG 0.5U	4	6.3	13.1	na	2.2	10.1	60.8	551
TG 5U	4	6.3	13.2	na	2.1	6.7	45.8	448
H-TG 5U	4	6.5	9.6	na	9.8	40.7	107.0	381
H-amylase	2	na	9.4	na	na	na	na	20
H-amylase TPG	2	na	80.3	na	na	na	na	352
H-amylase TG	4	na	8.2	na	na	na	na	25

* Viscosity was measured after 15 min heating at 95 °C, followed by cooling to room temperature.

Abbreviations: OPC = oat protein concentrate, TG = transglutaminase, TPG = combined transglutaminase and protein-glutaminase, t = enzyme treatment time in hours

In the current study, particle size measurements were detecting only insoluble particles due to the constraints of the analysis method in aqueous environment. However, it was possible to compare the results of the control and transglutaminase treatments, given their similar low solubility values. It was observed that the control treatment resulted in an increased particle size, which was further increased by the additional pre-heating step. This increase suggested that hydrophobic sites of the proteins were exposed, which led to protein aggregation (Nivala et al., 2020). In addition, also starch gelatinisation can increase the particle size after heating (Singh et al., 2023). Furthermore, heating breaks hydrogen bonds present both in proteins and starch, which can accelerate the interactions between them upon cooling (Cornet et al., 2022; Scott and Awika, 2023). Transglutami-

nase-mediated crosslinking led to a gradual decrease in particle size as a function of increased dosage when compared to the control treatment. Similar observation was reported by (Nivala et al., 2017), who observed a decreasing particle size after transglutaminase treatment of faba and oat protein isolates. However, they only measured the particle size of soluble fraction using a different analysis method compared to the current study.

The wide variation in solubility values after the combined treatment of trans- and protein-glutaminase at various dosages (0, 0.5, and 5 U) made the comparison of particle sizes difficult within this particular sample group. Despite the observed decrease in particle size with increasing dosage, there was a simultaneous increase in protein solubility. Therefore, it is unlikely that the protein size decreased after the treatment, rather, the larger particles became soluble. This observation is particularly noteworthy when considering previous research that reported an increasing trend in particle size as a function of the increasing deamidation degree of soy proteins, measured using a method capable of detecting particles that solubilise in water (Jiang et al., 2022). This indicates that the current study could also show an increasing trend in particle size (looser structure) with an appropriate analysis method detecting also the solubilised particles. The size of oat starch particles typically varies from 2-30 μm (Rostamabadi et al., 2022; Tian et al., 2016), which is in similar range compared to the residual particle size in water for the samples treated with the combined enzyme. This also suggested that the proteins that became soluble likely had larger particle sizes, leaving primarily the smaller, insoluble starch particles detectable for the used analysis.

All treatments of OPC involving the use of transglutaminase, or a combination of trans- and protein-glutaminase, with or without pre-heating, exhibited crosslinking. This crosslinking was observed as the formation of high-molecular-weight proteins in the loading wells of SDS-PAGE gels in Publication II. Moreover, the effect of protein-glutaminase was observed as a minor increase in the molecular weight of various protein bands. This increase was a result of the deamidation reaction, which converts glutamine and asparagine into higher molecular-weight residues of glutamic acid and aspartic acid, respectively (Gouseti et al., 2023; Zhang et al., 2021a). However, the observed crosslinking and deamidation only promoted the formation of fibrous structure formation under specific conditions that involved pre-heating.

Figure 10 provides a visual representation of how different treatments influenced the appearances of fibrous structures within the extrudates when bent across and along the extrudates. Although the native, control and pre-heating treatments demonstrated diverse structures, none of them exhibited fibrous alignment. The tensile strength values, of these samples, as presented in Table 21, were somewhat comparable, corresponding to values of 20-57 kPa in longitudinal direction and 51-55 kPa in perpendicular direction. The viscosity of the native and control raw materials of OPC varied between 695-539 cP, as shown in Table 6. These somewhat high viscosity values were attributed to starch, as the α-amylase treatment resulted in a notably lower viscosity of only 20 cP.

The combined treatment of trans- and protein-glutaminase at the lower dosage had minimal impact on structure, which was also seen as a minor change in tensile strength. However, a more rigid structure was observed with the higher dosage, with a shift of longitudinal tensile strength to a higher value (55 kPa) and perpendicular strength to a lower value (42 kPa). Interestingly, the addition of a pre-heating step before the enzymatic treatment significantly enhanced the structure, revealing the presence of fibres while bending.

Figure 10. Extrudate structures of oat protein concentrate (OPC) following various control and enzymatic treatments. Pre-heating = H, transglutaminase =TG, TPG = combined trans- and protein glutaminase, and α-A = and α-amylase (2 mg/g OPC). Dosages for TG and TGP were 0.5 and 5 enzyme units (U) per gram of OPC.

This was consistent with the notable increase in tensile strength values up to 114 kPa in the longitudinal direction and 109 kPa in the perpendicular direction. Furthermore, this was associated with a notable increase in the viscosity of the treated OPC, rising from the pre-heated control value of 539 cP to 1073 cP. Starch degradation decreased this viscosity to 352 cP accompanied with a loss of fibrous structure. This may be attributed to protein unfolding during heating and its subsequent aggregation that has been shown to influence starch functionality observed as an increased viscosity after heating a batter involving denatured and crosslinked gliadins within wheat starch matrix (Scott and Awika, 2023). Ad-

ditionally, Bühler et al. (2022) demonstrated the benefits of pre-gelatinised starch on fibrous structure formation of faba protein concentrate during HMEP, unable to form the fibres without the pre-gelatinisation.

The benefits of pre-heating have been previously reported by Nivala et al. (2020), who observed a positive effect of pre-heating prior to transglutaminase treatment on the gel formation ability of faba protein isolate. Furthermore, Jiang et al. (2022) also reported a beneficial acceleration effect of pre-heating on enzymatic deamidation through which they were able to improve emulsifying properties of soy proteins. However, in the current study, the benefits of pre-heating were only observed when using the combined enzyme of trans-and protein-glutaminase. The extrudate structure became crumblier when transglutaminase was used alone, especially at the higher dosage, and this loss of uniform structure was even more pronounced when combined with pre-heating. This observation aligned with the decreased tensile strength results in the longitudinal (38-64kPa) and perpendicular directions (33-40 kPa). The viscosity of transglutaminase-treated OPC with or without pre-heating varied from 381-551 cP and decreased to 25 cP after the amylase treatment. The starch degradation in combination with transglutaminase led to a loss of firm structure, observed as fragile and crumbly extrudates.

Similarly to the observations regarding the particle size, the free thiol group results in Table 21 suggested that the control treatment at 40 °C without enzymes, induced protein aggregation (prior to extrusion). This aggregation most likely resulted through non-covalent interactions, as the treatment temperature was low. In non-reducing conditions the free thiol groups of the control raw materials decreased compared to the native OPC, but once urea was applied to interrupt the non-covalent interaction, the control values increased to similar levels with the native OPC. This also suggested that the interactions were non-covalent and made the free thiol groups undetectable during the measurement in non-reducing conditions. Pre-heating further decreased the level of free thiol groups. However, when urea was added, the value remained low compared to the control, suggesting that the reaction involved the formation of disulfide bonds.

Table 21. Tensile strength and free thiol groups of native and modified oat protein concentrate (OPC). Different letters from a to f denote significant differences ($p<0.05$) between different samples within each analytics.

Sample	Tensile strength (kPa)		Free thiol groups (µmol/g)			
	P	L	RM (NaP)	RM (Urea)	E (NaP)	E (Urea)
Native OPC	51 ^{abc}	20 ^{a}	3.8 ^{a}	6.4 ^{a}	4.4 ^{a}	3.1 ^{a}
Control	55 ^{bc}	57 ^{cd}	1.5 ^{de}	5.8 ^{b}	3.2 ^{d}	2.4 ^{bc}
H-control	52 ^{abc}	54 ^{cd}	1.4 ^{de}	3.4 ^{de}	4.0 ^{bc}	2.4 ^{bc}
TPG 0.5 U	68 ^{c}	53 ^{c}	1.8 ^{d}	5.5 ^{b}	3.4 ^{d}	2.3 ^{c}
TPG 5 U	42 ^{ab}	66 ^{d}	2.2 ^{bc}	5.9 ^{ab}	3.0 ^{de}	2.7 ^{b}
H-TPG 5 U	109 ^{d}	114 ^{e}	1.8 ^{cd}	3.5 ^{de}	3.9 ^{c}	2.6 ^{bc}
TG 0.5 U	92 ^{d}	65 ^{d}	2.2 ^{b}	5.5 ^{b}	3.1 ^{de}	2.4 ^{bc}
TG 5 U	33 ^{a}	38 ^{b}	1.3 ^{e}	4.5 ^{c}	2.8 ^{ef}	2.3 ^{c}
H-TG 5 U	40 ^{ab}	64 ^{cd}	1.3 ^{e}	3.2 ^{e}	4.4 ^{ab}	2.7 ^{b}
H-amylase	na	na	1.3 ^{e}	3.8 ^{d}	2.7 ^{f}	2.3 ^{c}
H-amylase TPG 5 U	na	na	1.8 ^{abc}	3.8 ^{de}	3.0 ^{def}	3.1 ^{a}
H-amylase TG 5 U	na	na	1.3 ^{e}	3.7 ^{de}	1.9 ^{g}	3.3 ^{a}

Abbreviations: H = pre-heating for 15 min at 95 °C, TPG = combined transglutaminase and protein-glutaminase, TG = transglutaminase, U = enzyme units / g OPC, P = perpendicular, L = longitudinal, na = not analysed.

Treatments using either a combination of trans- and protein-glutaminase or transglutaminase alone, with or without pre-heating, resulted in varying outcomes before extrusion. The combined enzyme treatment resulted in higher number of free thiol groups, while

using only transglutaminase resulted in lower level of free thiol groups when compared to the controls. This suggested that the combined enzyme treatment resulted in a looser protein structure, while transglutaminase alone at higher dosage produced a denser structure, a finding consistent with the particle size results. When urea was applied, it was discovered that the thiol groups decreased only when the pre-heating step was applied or when transglutaminase was used alone at the higher dosage. This suggested that the changes in protein interactions were mainly disulphide bond-mediated when the pre-heating or transglutaminase alone was applied. Regarding transglutaminase, the crosslinking through glutamine and lysine residues (Gouseti et al., 2023) within one protein molecule can make the protein more compact. This compactness might conceal the free thiol groups within the protein core, leaving them undetectable by Ellman's reagent during the measurement due to steric hindrance. On the other hand, as the protein-glutaminase acts on glutamine residues and converts them to glutamic acid, there are fewer amino acids available for crosslinking when the combined deamidation and crosslinking treatment is applied. This increases the likelihood of the protein to forming intermolecular interactions instead of intramolecular ones. This would explain why fibrous structure formation of OPC requires the combined enzyme instead of transglutaminase alone.

After extrusion, the number of free thiol groups increased in all extrudates compared to their raw material counterparts. This suggested that extrusion led to a significant rearrangement of the protein structures (Cornet et al., 2022; Sun et al., 2022; Zhang et al., 2022b), thereby exposing number of free thiol groups. Interestingly, the application of urea (reduces the non-covalent bonds including hydrogen bonds) in extrudate samples led to a decrease in the number of free thiol groups. This implied that once the non-covalent bonds (including hydrophobic interactions and hydrogen bonds) were reduced by urea, the existing free thiol groups became reactive and started to form disulphide bonds during the measurements (Ryle and Sanger, 1955). However, two exceptions were observed in the samples that underwent combined pre-heating and starch degradation (α-amylase treatments) with either transglutaminase alone or with the combined trans- and protein-glutaminase. This observation indicated that the presence or absence of starch may play a significant role in this phenomenon when combined with crosslinking and deamidation. To shed light into this matter, it is important to note that the main interactions between protein and starch are hydrogen bonds, which suggested that when these bonds were reduced by urea in extrudate samples, the free thiol groups inside protein-starch matrix became exposed and started to react during the measurement leading to a reduction in free thiol groups. This observation aligned with the structural changes seen in α-amylase treated OPC, in Figure 10. There was a notable shift from a rigid and uniform (non-fibrous) structure to a crumbly appearance once crosslinking or a combination of crosslinking and deamidation was applied.

Research on of starch-protein interactions in the HMEP is somewhat limited. However, a study by Chen et al. (2022) provided evidence on an increased degree of fibrousness when 10% amylopectin, a primary component of starch, was added to pea protein isolate during HMEP. They found that the addition of amylopectin promoted unfolding of pea protein chains in the extruder die, which facilitated protein rearrangement in the cooling phase, thereby promoting the formation of fibrous structures. In the current study, the fibrous structure, which resulted after the combined treatment of pre-heating with trans- and protein-glutaminase, was lost after starch degradation. This observation suggested

that the starch-protein interactions were crucial for structure formation in the modified OPC. Building on this, it is important to note that heating can induce starch retrogradation, which can be affected by proteins (Scott and Awika, 2023). Starch and proteins interact with each other through hydrogen bonding which can be facilitated by heating. In this study, deamidation further facilitated this interaction, as it can also uncoil the protein structure (Fang et al., 2020; Jiang et al., 2022; Liu et al., 2011; Shen et al., 2022). On the other hand, deamidation is facilitated when the protein is slightly uncoiled by a pre-heating step (Jiang et al., 2022). Simultaneously, as the protein and starch interactions increase through hydrogen bonding, it forces the protein to adopt a more open structure, which facilitates the transglutaminase-mediated crosslinking between glutamine and lysine residues.

The impact of starch degradation on protein and combined trans- and protein-glutaminase interactions was evident also in the changes observed in protein patterns on SDS-PAGE gels in Figure 11. Somewhat uniform protein patterns were resolved to the gel for both unmodified and modified OPC samples and extrudates when starch was intact, even with pre-heating. However, changes occurred when samples were pre-heated, starch was degraded, and proteins were enzymatically crosslinked or simultaneously crosslinked and deamidated. Specifically, when pre-heating, starch degradation, deamidation, and crosslinking were simultaneously applied, a globulin band appeared in the extrudate lane under non-reducing conditions (intact disulphide bonds). However, when transglutaminase was applied alone along with the pre-heating and starch degradation, the globulin band of the extrudate was even more pronounced. Under reducing conditions (disulphide bonds disrupted), both of these treatments revealed β-subunit bands for the extrudate samples, which were not visible when starch was intact. Moreover, under the non-reducing conditions, both β- and α-subunit bands became visible only in the transglutaminase treated OPC raw materials.

This suggested that when transglutaminase was used alone, it heavily catalysed the lysine-glutamine crosslinking within a single globulin molecule of OPC or its subunits, leading to a reduction in particle size without affecting the molecular weight. Therefore, as this protein resolved to the SDS-PAGE gel as a clear band under non-reducing conditions, it suggested that it was not participating in the intermolecular disulphide bond-mediated protein network required for fibrous structure formation. Thus, the weak structure observed for the extrudate in Figure 11 was mainly held together through non-covalent interactions, as was also suggested by the thiol results. To conclude, as the globulin band appeared less intensive when the enzymatic reaction involved also deamidation, it played a key role in preventing excessive intramolecular transglutaminase-mediated crosslinking between lysine and glutamine residues.

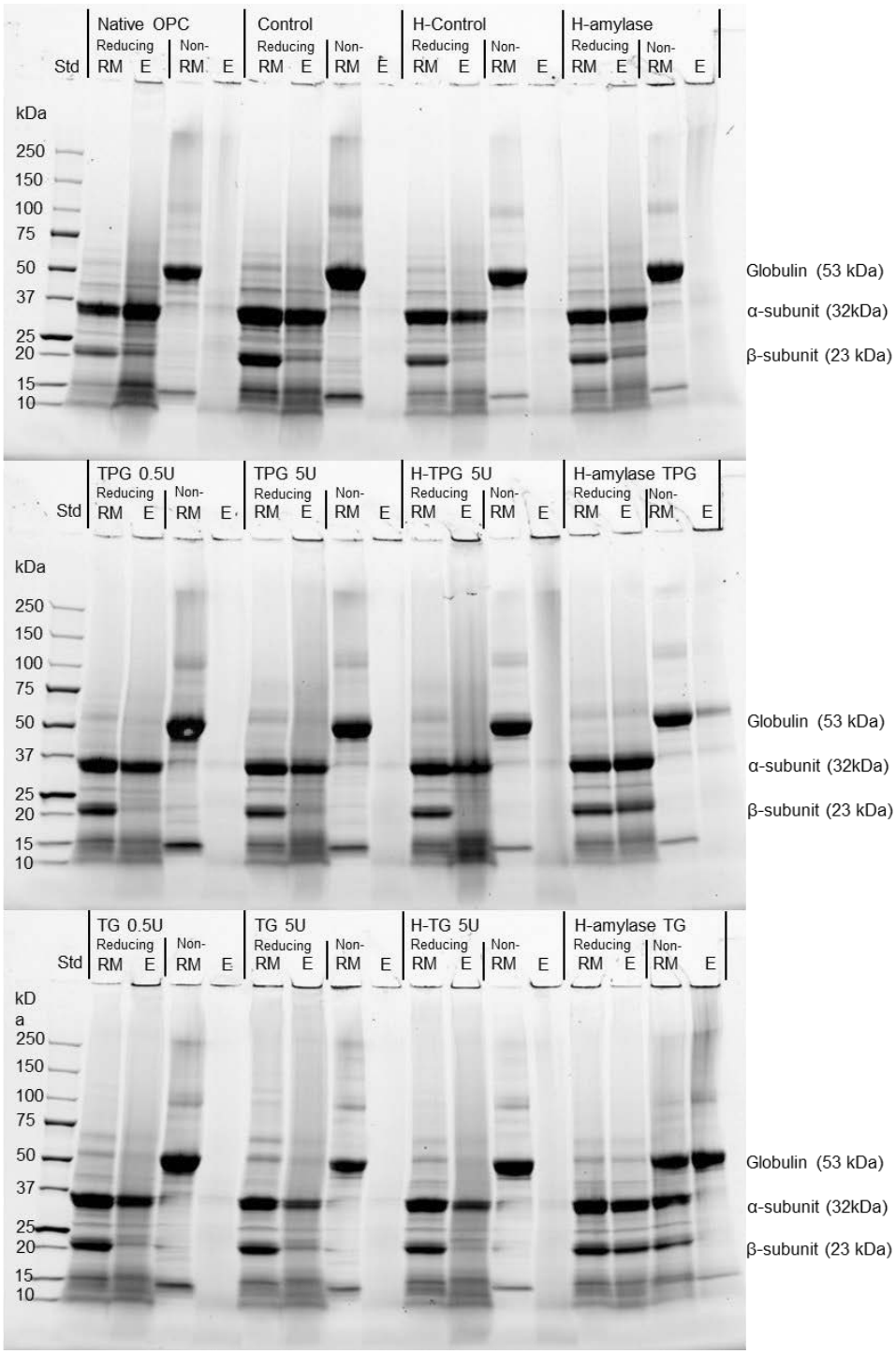


Figure 11. Protein profiles of oat protein concentrate (OPC) after several treatments including control, pre-heating (H), α-amylase, transglutaminase (TG), combined trans and protein-glutaminase (TPG) using 0.5 U and 5 U /g OPC dosages. RM refers to the enzymatically treated sample before extrusion while E denotes the sample after extrusion in reducing or non-reducing (= non-) conditions.

To the best of available knowledge, no prior studies have investigated the combined effect of trans- and protein-glutaminase on any plant proteins during HMEP. This makes comparison with other research difficult, especially given the lack of research on oat protein ingredients as a single ingredient in fibrous structure formation. Furthermore, the complexity increases as OPC includes high amount of starch that clearly affected the resulted outcomes of the enzymatic modifications. However, one prior publication regarding the use of transglutaminase alone (without pre-heating) on peanut protein concentrate during HMEP exists, reported by Zhang et al. (2021b). In contrast to this study, they reported an increase in fibrous degree as a result of transglutaminase treatment alone as a function of increasing dosage. However, the absence of visual evidence in their publication made comparison challenging. A subsequent study by Zhang et al. (2023) extended the investigation on a variety of plant proteins such as, soy, peanut, pea, wheat and rice, yielding diverse outcomes. For instance, while the treatment showed no impact on rice proteins and reduced the fibrousness of wheat gluten, it showed considerable improvement in fibrous structure formation in pea protein isolate. This raw material dependency could be one plausible explanation of why transglutaminase alone was not able to improve the structure formation of OPC during HMEP. Furthermore, they used lower dosages, which could hinder the observed excessive intramolecular crosslinking of OPC in the current study.

In conclusion, the successful formation of fibrous structure in low-protein OPC required a combined treatment involving pre-heating, deamidation and crosslinking. The formation of this fibrous structure was a complex interplay between starch and proteins requiring specific conditions for achieving an optimal level of starch-protein interactions and protein uncoiling by pre-heating and subsequent deamidation simultaneously with crosslinking reaction.

5.3.3 The effect of peptide formation through fermentation on fibrous structure formation and sensory characteristics

The unmodified RPC (IV), produced through dry fractionation, was a low-protein (41%) raw material rich in dietary fibre (27%). It showed intermediate protein solubility in water (36 %), and relatively good fibrous structure formation ability in HMEP (Figure 8). However, in line with the typical characteristics of ingredients derived from rapeseed, this was also associated with an intense bitterness along with a chemical and pungent flavour.

Therefore, this study aimed to improve its sensory properties through fermentation using strains such as *L. plantarum* and *W. confusa*, which are known for their ability to improve structure and flavour properties. The primary finding was that fermentation induced a loss in fibrous structure, later associated with protein hydrolysis. As demonstrated in Publication II, the fibrous structure formation was greatly affected by pH. Consequently, Publication IV investigated a diverse range of control samples using pH-shifting and incubation without a starter culture. This approach aimed to evaluate the factors contributing to the observed structural loss. Figure 12 presents the effect of varying fermentation and control treatments on extrudate structures.

A specialised structural terminology (see Publication IV for more details) was developed to describe the resulted extrudates, utilising the sensory results, visual appearance, and tensile strength (Table 22) measurements. Consequently, descriptive terms excellent, good, medium, poor, and no structure were selected to be associated with the correspond-

ing extrudates (Figure 12). The native extrudate exhibited a good structure with lamellar type of fibrousness. All fermented (with starter culture) and incubated (no starter culture) extrudates exhibited a moderate, poor or no structure under acidic conditions (pH 4-5) regardless of the attempt to readjust the pH back to 6. Only the non-incubated control extrudates with subsequent readjustment of pH to 6 exhibited excellent fibrous structures. The difference between a good and excellent structure was differentiated in terms of fibre type, with excellent structures exhibiting thin fibres instead of lamellar ones.

The sensory evaluation of the resulted extrudates after HMEP demonstrated that the native RPC extrudates exhibited slight bitterness, quite intense chemical flavour and odour, as well as pungent radish-like flavour. Compounds associated with these flavour-related attributes have been detected in various rapeseed protein ingredients. Zhang et al. (2024) identified compounds, including sulphide, nitrogen, carbonyl, and organic solvents, among others, which were linked to sensory attributes such as bitterness and astringency. Additionally, one of the major compounds identified as the cause for bitter taste in rapeseed protein isolates has been reported to be kaempferol 3-O-(2‴-O-Sinapoyl-β-sophoroside) as reported by Hald et al. (2019).

The sensory analysis in the current study showed that the chemical odour and flavour, detected in the unmodified RPC extrudates, were decreased by the fermentation process (see the results in publication IV, Figure 1). Several studies using *L. plantarum* strains have reported improved sensory properties of pea proteins through decreased bitterness, beany odour and flavour, as well as increased umami flavour (Shi et al., 2021; Valtonen et al., 2023). However, in the current study the fermentation induced bitterness, sourdough flavour, mustard-like odour, rye bread odour, doughy mouthfeel, and stickiness along with a loss in biting resistance and a decreased number of visible fibrils while bending. This was accompanied by a decrease in the fibrous structure from a good to no structure, which corresponded to a decrease in tensile strength from the original 24 and 56 kPa to 15 and 17 kPa in the longitudinal and perpendicular directions, respectively. In accordance with the findings of this study, Pöri et al. (2023) reported an increased bitterness and decreased number of fibrils in extrudates produced from a mixture of fermented sunflower concentrate and pea protein isolate during HMEP. Similarly, another study by Kaleda et al. (2020), which used dry extrusion to produce fibrous structures in fermented blend of pea and oat proteins, also reported a negative impact of fermentation on structure formation.

In the current study, the control treatments that shifted the pH to an acidic environment of pH 4 using lactic acid with or without incubation reduced the chemical flavour and odour but did not increase bitterness. However, these control treatments in acidic environment, especially the one involving incubation step, led to an increase in stickiness and doughy mouthfeel, along with a loss in biting resistance and number of fibrils. This observation was consistent with the visual classification of no structure. The control at pH 4, which was adjusted using a different stronger acid, namely hydrochloric acid, resulted in tensile strength values almost identical to the fermented counterpart and was similarly classified as having no structure.

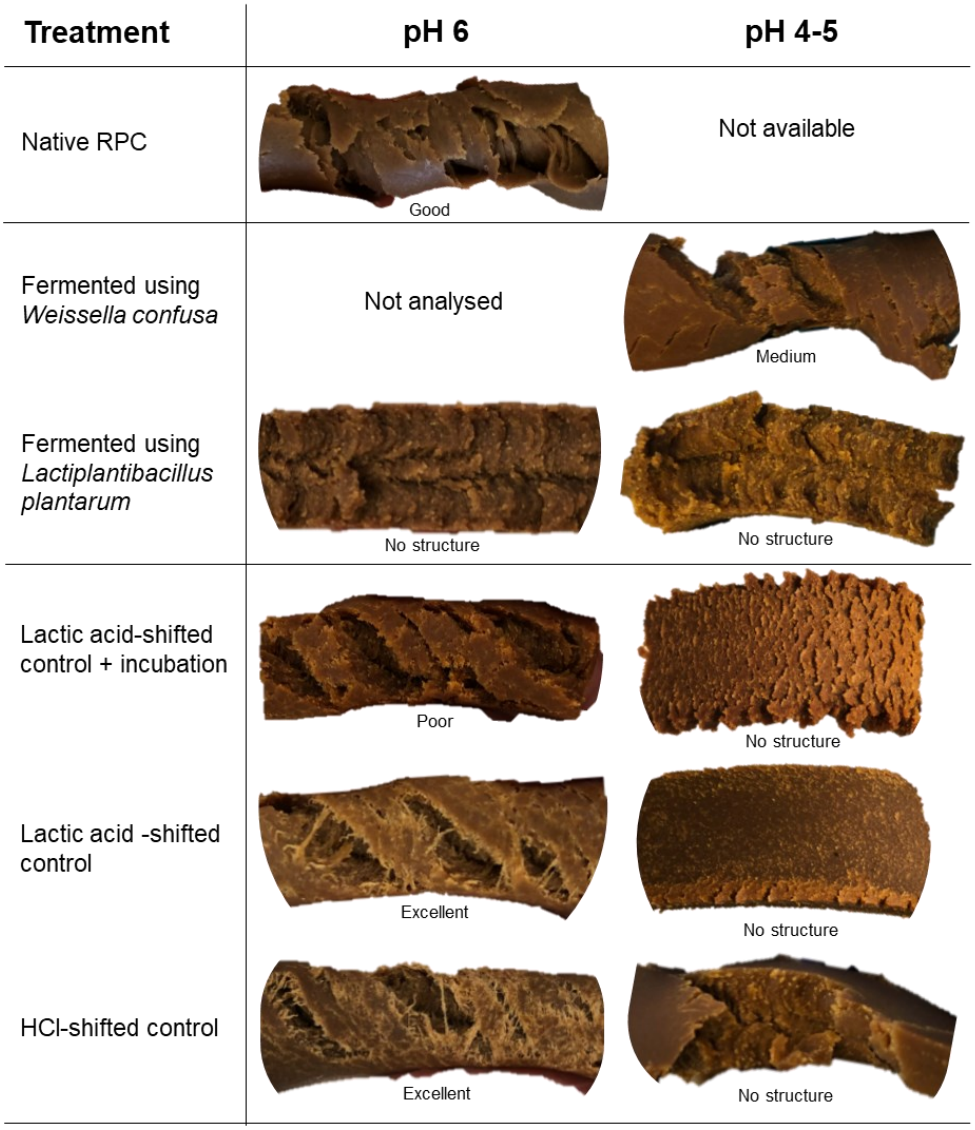


Figure 12. Effect of fermentation and pH-shifting on macrostructure of rapeseed protein concentrate (RPC) visualised as digital images. Fermentation and incubation conditions were identical (24 hours at 25 °C)

When the sample fermented with *L. plantarum* was returned to a pH of 6, its bitterness and radish-like flavour decreased, along with sensory attributes that were induced by fermentation, such as vinegar odour and sourdough flavour. However, regardless of the pH shift towards neutral conditions and its beneficial effect on flavour and odour attributes, the structural loss remained unchanged. A slight increase in tensile strength was noted, but it was not statistically significant ($p < 0.05$). Interestingly, in contrast to findings in the current study, Pöri et al. (2023) reported that shifting the pH towards neutral conditions restored the fibrous structure. In addition to the different raw material in their study, these contradictory outcomes could be associated with the use of a different strain, namely *Lactobacillus helveticus*. In this study, the fibrous structures were only restored after acidification to pH 4, when the incubation and fermentation steps were excluded,

and the pH was shifted back to 6. This resulted in structures classified as excellent and were accompanied with the statistically ($p < 0.05$) significant increase in tensile strength values of 40 kPa and 52-63 kPa in longitudinal and perpendicular directions, respectively.

The *W. confusa* strain, which acidified the RPC to pH 5, resulted in a less detrimental effect on the fibrous structure, as evidenced through visual inspection (Figure 12) and confirmed through sensory analysis. For instance, while the biting resistance and number of fibrils decreased compared to the native RPC, they were notably higher than those in the *L. plantarum* samples. Similarly *to L. plantarum*, *W. confusa* was successful in reducing the chemical flavour and odour, while it resulted in higher values for bitterness and. The relatively minor impact on the structure could be attributed to the slightly higher pH value at the end of the fermentation. On the other hand, *W. confusa* is the well-known for its dextran-producing capabilities, which in turn, is known to enhance the structural properties of sourdoughs. For instance, Galli et al. (2020) demonstrated that W. confusa increased the dextran levels during fermentation, thereby increasing the final viscosity of chickpea-based sourdough. Similarly, Wang et al. (2022) reported increased viscosity in rapeseed protein-based sourdoughs after fermentation with *W. confusa*. Given the major role of protein-polysaccharide interactions in structure formation in Publication III, similar phenomena could be protecting the rigid structure of *the W. confusa* extrudate in this study.

While the afore-mentioned two studies by Kaleda et al. (2020) and Pöri et al. (2023) have also observed the negative impact of fermentation on fibrous structure, the underlying mechanism has remained unexplored. Considering previous research by Lee et al. (2019) reporting that different *L. plantarum* strains have extracellular proteolytic activities, it is likely that these enzymatic activities contributed to the structural loss observed in this study fermented RPC extrudates. Additionally, Wang et al. (2022) reported an increased protein hydrolysis after *W. confusa* fermentation in rapeseed proteins. Moreover, considering that RPC raw material in this study was produced through cold-pressing followed by dry fractionation, it is possible that it has retained some endogenous enzymes activities originated from the rapeseed plant. For instance, Chen et al. (2021) have reported the presence of endogenous exo- and endoprotease activities in peanuts, another type of oilseed, specifically under acidic conditions within a temperature range of 10-60 °C. These possible enzyme activities, originating both from the starter culture and the plant itself, may have been particularly activated during the fermentation and incubation conducted at 25 °C for 24 hours. Additionally, the incubation process was initiated under acidic conditions, and as the fermentation process progressed the pH level gradually decreased.

Given these facts, evidence for proteolytic degradation was investigated through peptidomics analysis (3-4600 kDa), free amino ends and solubility analysis (Table 22), as well as protein profile analysis visualised by SDS-PAGE gels. The peptidomics results, presented in Publication IV, demonstrated an increased presence of peptides particularly in samples that had underwent incubation or fermentation treatments. Similar findings regarding fermentation of rapeseed protein concentrate were reported by Wang et al. (2019) who demonstrated a significant increase in peptides after a combined treatment with lactic acid bacteria and yeast. Additionally, in the current study, acidic conditions showed a higher relative number of peptides compared to pH 6, particularly when the stronger acid (hydrochloric acid) was used. This explains the detected bitter taste in fermented and incu-

bated samples as it has been widely studied that peptides produced during fermentation can induce bitter taste (Zhao et al., 2016).

Table 22. Effect of fermentation (24 hours at 25 °C), incubation (24 hours at 25 °C), and pH-shifting on fibrous structure and related physicochemical properties of rapeseed protein concentrate.

Sample	Final pH	Solubility (%)		Free amino ends (mg/mL)		Free thiol groups (μmol/g)		Sensory (structural)		Tensile (kPa)		Fibrous degree
		RM	E	RM	E	RM	E	Fibrils*	Biting*	P	L	
Native	6.1	36^{h}	8.4^{r}	2.4	1.9^{de}	27.2	19.7	8.4	6.4	56^{a}	34^{a}	Good
f-WC	5.2	61^{b}	30^{l}	3.9^{j}	3.0^{a}	18.6	18.7	5.2	5.9	na	na	Medium
f-LP-N	6.0	69^{a}	30^{k}	3.7^{j}	2.7^{b}	18.3	16.0	2.6	4.1	26^{b}	21^{b}	No
f-LP	4.2	47^{e}	32^{j}	3.5	2.5^{b}	18.6	17.1	3.0	3.8	17^{b}	15^{b}	No
C-i-la-N	6.1	58^{c}	28^{m}	3.1	2.0^{cde}	16.1	15.4	na	na	na	na	Poor
C-i-la	3.8	39^{g}	34^{i}	2.9^{j}	2.3^{bc}	16.5	14.6	2.9	2.4	36^{b}	21^{b}	No
C-HCl-N	6.1	44^{f}	14^{q}	2.7	2.0^{cde}	19.1	15.9	na	na	63^{a}	40^{a}	Excellent
C-la-N	6.1	48^{d}	15^{p}	2.3	1.7^{e}	17.9	15.2	na	na	52^{a}	40^{a}	Excellent
C-HCl	4.1	29^{l}	24^{n}	2.2	2.1^{cd}	18.1	16.1	na	na	21^{b}	15^{b}	No
C-la	4.1	24^{n}	19^{o}	2.1	1.8^{de}	18.7	15.6	3.2	3.3	na	na	No

Statistical analysis: significant differences ($p < 0.05$) are denoted by superscript letters within the following groups: All solubility samples, RM samples in free amino end results, and tensile strength results within P and L groups separately. The significant difference in values within certain sensory attribute is marked with *. Free amino end results for RM were compared pair-wise to the native sample where j denotes the significant difference. Free thiol groups were compared group-wise: All RM samples differed significantly ($p < 0.05$) from its counterpart E, except for f-WC sample.

Abbreviations: f = fermentation, LP = *Lactiplantibacillus plantarum*, WC = *Weissella confusa*, N = pH shifting using NaOH, C = control treatment, i = incubation without starter culture, la = pH-shifting using lactic acid, HCl = pH-shifting using hydrochloric acid, RM = sample before extrusion, E = extrudate, P = perpendicular, L = longitudinal.

The free amino end results in the current study supported the results of the peptide formation, evidenced as significant increase ($p < 0.05$) in number of free amino ends during fermentation and incubation. Additionally, the stronger acid, namely hydrochloric acid, resulted in a slight increase in free amino ends although it was shifted to pH 6, but this was also seen in peptidomics results. This suggested that the mechanism of acid hydrolysis differs from the one induced by incubation and fermentation and was not detrimental for fibrous structure formation. A simultaneous increase in solubility was observed with increasing number of free amino ends, which aligned with previous research on protein hydrolysis. For instance, several studies on wheat and rice proteins have reported a positive correlation between an increasing protein solubility and increased degree of hydrolysis (Kong et al., 2007; Paraman et al., 2007; Xu et al., 2016).

The highest number of free amino ends was observed for *W. confusa* samples along with a notably high solubility of 61% in acidic environment, close to the isoelectric point of rapeseed proteins. This indicated that *W. confusa* samples underwent the most intensive protein hydrolysis. These findings highlight the proposed mechanism of protein-polysaccharide interactions acting as a protective reaction against structural loss. Furthermore, these observations were consistent with the free thiol group results that evidenced significant changes between the treated raw materials and their corresponding extrudates with one exception regarding the *W. confusa* samples that exhibited identical values before and after extrusion. This phenomenon suggested the absence of disulfide bond formation during HMEP. Similarly to this study, Zhu et al. (2008) reported that dextran could act as a protective agent against excessive protein denaturation and aggregation when it produces conjugates with whey proteins.

The protein profiles on SDS-PAGE, seen in publication IV, did not clearly show any peptide formation, suggesting that all the produced peptides had sizes under 10 kDa (detection limit). This was in line with peptidomics results that were set to detect smaller than 10 kDa peptides. However, few aspects in protein profiles correlating with the structure loss

could be noted. For instance, a clear variation in the intensity of a certain polypeptide band in extrudates, identified as the α-polypeptide chain of cruciferin, was noted under reducing conditions (disulphide bonds interrupted). The intensity exhibited a positive correlation with both elevated pH conditions and a reduced number of peptides detected in peptidomics results. In all samples where the α-subunits were absent, the fibrousness was classified as no structure. The visibility of α-subunit bands under the reducing conditions, indicated that it contributed to structure formation through disulphide bonding. On the other hand, when the band was absent from the protein profiles, it suggested that they may have been forming protein complexes with non-protein molecules. This could possibly be through other types of covalent bonds, given that disulphide bonds are the only known covalent interactions during HMEP. The varying intensities observed across different samples suggested that these interactions may have occurred simultaneously.

One plausible explanation for the complexation could be that these subunits have interacted with antinutrients such as phenolic compounds, phytic acid, or hydrolysis products of glucosinolates. These antinutrients, known to be present in rapeseed, tend to form complexes with proteins (Kies et al., 2006; Kroll et al., 1993; Naczk et al., 1998a, 1998b). Among these compounds, the hydrolysis products of glucosinolates, namely isothiocyanates, can form covalent interactions with free amino and thiol groups of proteins (Kroll et al., 1993). Phenolic compounds can form non-covalent interaction through hydrogen bonding and covalent interactions through their oxidised quinone structures particularly with nucleophilic amino acid residues including cysteine (Naczk et al., 1998b). Phytic acid is only known to form non-covalent interactions with proteins through electrostatic interactions and hydrogen bonding (Serraino and Thompson, 1984). Furthermore, these complexes often exhibit a strong pungent and bitter flavour (Bell et al., 2018; Naczk et al., 1998a), which could explain the changes observed in sensory properties of the fermented extrudates in the current study.

However, this phenomenon requires further investigation in this context as the scope of Publication IV was to investigate protein hydrolysis. Despite the lack of analytics conducted on this matter in the current study, it is worth noting a few observations regarding free amino ends and glucosinolates. For instance, the reduction of observed free amino ends was double compared to the native and pH-shifted non-incubated samples, indicating increased covalent bonding with free amino ends, assuming that the reduction seen in the other samples was caused by steric hindrance restricting access of OPA reagent the target sites. Additionally, preliminary results (see Publication IV) indicated that glucosinolates degraded during fermentation with *L. plantarum*, suggesting myrosinase activity and resulting in isothiocyanates. Furthermore, it has been documented that myrosinase, an enzyme responsible for hydrolysing glucosinolates in rapeseed, can activate specifically under an acidic environment at a temperature of 25 °C (Aripin and Surugau, 2016), which was used as the incubation and fermentation temperature in the current study.

In conclusion, these results demonstrated the detrimental effect of acidification on fibrous structure formation during HMEP, whether through fermentation or incubation. It was discovered that after fermentation, even the pH-shift back to the original conditions failed to regain the fibrous structure of RPC extrudates. This research also suggested that different strains resulted in different outcomes. For instance, while *L. Plantarum* led to complete structural loss, *W. confusa* led to an intermediate structure formation. Overall, the fermentation was successful in reducing the chemical flavour, it induced bitterness,

which is not an acceptable sensory property. The chemical flavour was also reduced by the chemical pH-shift, without the subsequent introduction of bitterness. This finding suggested that pH shifting is more viable strategy for functionalisation of RPC for meat analogue applications.

5.4 Limitations of the study

5.4.1 Experimental design

It is important to mention that the goal of the current study was on finding processing solutions through case studies from independent projects that had varying goals, which dictated the overall scope of the research setup for this study. In other words, a systematic comparison of processing conditions and different functionalisation strategies for all raw materials was not possible. This emphasised the importance of comparing the modified raw materials specifically to their non-modified counterparts, rather than to well-performing reference raw materials. An exception was made in Publication II, where it was more logical to compare two distinct pH conditions across a wide range of raw materials, including the well-established PPI and WG references. In Publication II, the original pH values of the raw materials were all different, making direct comparison to the original states impractical. Expanding the experimental setup to include such comparisons would have exceeded the project budget.

Additionally, all treatments of this study involved water use followed by freeze-drying, making the process energy-intensive, less environmentally friendly, and unfeasible for industrial applications. Thus, it is important to highlight that this research does not propose these steps for industrial-scale functionalisation processes. Rather, the main purpose of this study was to demonstrate the potential of these strategies at a laboratory scale. However, these processes could be adapted for industrial scale in various ways. For instance, the pH-shifting could be performed directly during HMEP, eliminating the need for prior water additions, as recently demonstrated by recently demonstrated (Ellwanger et al., 2024). Similarly, there is evidence that the enzymatic treatments can be integrated into HMEP, as was demonstrated by (Zhang et al., 2021b). Alternatively, for dry fractionated protein concentrates, enzymatic processes could be conducted in a bioreactor with a high solid content and fed directly into the extruder as a pre-hydrated raw material. These approaches would exclude the excess water usage and the need for a drying step. In the case of wet-extracted protein isolates, these functionalisation steps could be integrated to the current manufacturing process.

Publication II could have investigated wider range of pH conditions. However, the original pH conditions of the raw materials varied notably, making it impossible to compare all raw materials to each other at their native pH levels. Instead, three preliminary pH conditions relevant for food applications (pH 5, pH 7, and pH 8) were selected. Initially, the native raw materials were extruded and compared to extrudates produced at these shifted pH conditions. Notably, the most significant structural differences were observed between pH 5 and 7, although fibrous structure continued to improve at pH 8. However, some raw materials, particularly WG, exhibited instability during HMEP at pH 8, leading to the exclusion of this pH level from further investigation.

Publication III included treatments using transglutaminase alone and a combination transglutaminase and protein-glutaminase. However, pure protein-glutaminase was not investigated due to availability issues. Future studies could explore the use of pure protein-glutaminase to determine if it alone is sufficient for improving fibrous structure formation. Additionally, the functionalisation approach with trans- and protein-glutaminase was only tested on starch-rich raw materials, and further research is needed on proteins deficient from starch to see if this process works also without the starch in other raw materials. Furthermore, the starch degraded extrudates were investigated in much lower HMEP temperatures due to their instability at higher temperatures. Future studies could explore further efforts to be able to produce starch-degraded extrudates at similar temperatures than the main samples.

Publication IV demonstrated how different strains affect structural outcomes, and literature suggested that also various raw materials lead to different results. Therefore, a broader range of strains and raw materials should be included in future studies to draw comprehensive conclusions about the effects of fermentation on structure formation ability.

5.4.2 Analytics

Publication I analysed some protein characteristics, such as surface hydrophobicity and zeta potential, only from the soluble fraction of the raw materials, while functional properties were analysed from the whole material. This limited the understanding of protein behaviour in the entire raw material and requires further investigation for a thorough understanding of functionalised REPI behaviour in foaming gelation, and colloidal stability applications.

Meat analogue-related studies investigated free thiol groups to provide insights into disulphide bond formation of the studied raw materials during HMEP. However, as was found by Zhang et al. (2022b), increasing the relative amount of intermolecular disulphide bonds is crucial for fibrous structure formation. Due to the lack of appropriate measurement techniques, the current study, was unable to distinguish whether the newly formed disulphide bonds during processing were intra or intermolecular.

Particle size measurements were conducted using a laser diffraction method in an aqueous environment to mimic the conditions during HMEP. However, different functionalisation methods led to varying solubility values, resulting in undetected soluble particles during measurements. Future studies could measure particle size in both wet and aqueous environments or use the methods that detect also the size of soluble particles.

Publication IV involved sensory profile analysis using a trained sensory panel. However, the related project was conducted during the COVID-19 pandemic, limiting the number of sensory evaluators and making it impossible to include all control samples in the sensory analysis.

6. Conclusions

The increasing global population and global warming have highlighted the need for sustainable and environmentally friendly alternatives to animal proteins. The consumption of animal-derived products, particularly from livestock, notably contributes to greenhouse gas emissions, deforestation, and high water usage. In response to these environmental concerns and the growing demand from health-conscious consumers for ethical and sustainable options, plant-based proteins have emerged as viable alternatives. However, plant proteins often exhibit limited functional properties compared to their animal counterparts, particularly in terms of solubility, foaming, gelation, colloidal stability, emulsification and fibrous structure formation.

The objective of this study was to address these challenges by enhancing the functionality of plant proteins for use in meat analogues and solubility-dependent applications. The study investigated a wide range of protein isolates (72-74% protein) produced through wet fractionation, as well as protein concentrates (40-49% protein) produced through dry fractionation, derived from rice, pea, wheat, oat, and rapeseed. This research used strategies such as limited protein hydrolysis, pH-shifting, enzymatic crosslinking, deamidation, and fermentation to modify the performance of these proteins in foaming, gelation, colloidal stability, and meat analogue applications. The fibrous structures for meat analogues were generated through HMEP, an industrially relevant production method.

The key findings indicate that the applied strategies are highly relevant for improving plant protein functionality, particularly for concentrates with low protein content that are usually unable to produce acceptable fibrous structures as a single ingredient. Specifically, limited hydrolysis with an intermediate degree of hydrolysis of 1.5% using neutral endoprotease was particularly effective in enhancing the foaming properties of rice proteins. Shifting the pH to neutral conditions was especially efficient at improving fibrous structure formation in PPC, as well as REPI, WG and PPI, associated with increased reactivity of disulphide bonding at elevated pH levels. The pH shift also reduced the temperature required for initiating the fibrous structure formation. Crosslinking and deamidation combined with pre-heating successfully improved fibrous structure formation in the low-protein oat ingredient during HMEP. Control studies using starch degrading enzyme indicated that starch-protein interaction played an important role in fibrous structure formation with the OPC. Although fermentation showed potential for reducing chemical off-notes in RPC extrudates, it induced proteolysis reactions that led to bitterness and loss of fibrous structures. Furthermore, the RPC control treatment using pH-shifting was able to reduce the chemical off-notes to similar level indicating that the pH-shifting alone is more effective functionalisation method as it also preserved the fibrous structure.

The results of this study suggest that applying the proposed strategies for plant-protein ingredients would notably increase their viability and utilisation in various ways. It would

expand the use of low-protein ingredients in meat analogues and otherwise insoluble plant proteins, to solubility-dependent applications such as plant-based drinks and egg replacement in baking. These strategies would also promote the broader use of more energy-efficient dry-fractionated raw materials, reducing production costs and further minimising the environmental impact of the food industry through improved resource efficiency and reduces decency on animal proteins. Furthermore, the possibility to use concentrates would benefit consumer health by incorporating dietary fibre, which is often lacking in western diets. Moreover, it supports consumer demand for more ethical and sustainable dietary options, aligning with health-conscious and environmentally aware lifestyles.

Despite the promising results of this study, there are limitations that need to be acknowledged. This study was designed to demonstrate the proof-of-concept at a laboratory scale, and the developed functionalisation strategies require further development for industrial-scale application to achieve the stated environmental benefits. Therefore, future research should focus on investigating the application of enzymes or pH-shifting directly through HMEP, which would eliminate the need for excessive water use and drying. Alternatively, high solid content treatments in bioreactor prior to extrusion, using only the water required for meat analogue production, could be explored. For protein isolates that already utilise water in their production process, the pH-shifting and enzymatic treatment could be incorporated to the current processes. Regarding the fermentation, a wider range of strains and other raw materials should be explored to draw final conclusions on the relevance of the process for meat analogue applications. Furthermore, techno-economic and life cycle analyses should be conducted to be able to verify the environmental and economic feasibility.

In conclusion, this study demonstrated that the explored functionalisation strategies can enhance the applicability of plant proteins if food products, facilitating the transition towards more sustainable and healthier dietary options. By identifying promising functionalisation methods, this research contributes to the development of solutions that support both industry needs and consumer preferences, eventually facilitating the development of a more sustainable food system.

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This study aims to unleash the potential of plant proteins in meat analogues and solubility-dependent applications, driven by the need to reduce reliance on animal proteins for environmental reasons. It particularly focuses on strategies that enable protein concentrates, not just isolates, to be crafted into fibrous structures. Additionally, it addresses the functionalisation of insoluble wet-extracted protein isolates. Strategies include controlled enzymatic hydrolysis, deamidation, and crosslinking, and pH-shifting through fermentation and chemical methods. By enhancing plant protein functionality, this research supports development of sustainable food alternatives.

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