FACULTEIT
BIO-INGENIEURSWETENSCHAPPEN

COMBINING STRATEGIES FOR OPTIMAL PROTEIN EXTRACTION FROM YELLOW PEA HULLS

Anouk Wijffels
Student number: 02010665

Promotor: Prof. Dr. Ir. Katleen Raes
Tutor: Ir. Ben Van den Wouwer

A dissertation submitted to Ghent University in partial fulfillment of the requirements for the degree of Master of Science in Bioscience Engineering Technology: Food Industry

Academic year: 2023 – 2024

UNIVERSITEIT
GENT

PREFACE

This thesis was written to conclude my studies in bioscience engineering at Gent University, and focuses on enhancing the protein extraction yield from yellow pea hull. The subject of the valorization of by-products from the food industry was quite new to me since no courses focused on this topic besides a couple of small chapters. Luckily, I had help along the way. Therefore, I want to express gratitude to the following people:

Firstly, I would like to thank my promotor, Prof. Dr. Ir. Katleen Raes, for her dedicated overall supervision, and insightful suggestions that helped finalizing this thesis successfully throughout the year. Secondly, I would like to thank my tutor, Ir. Ben Van den Wouwer, for the sound mentorship over the past year. The various procedures were always clear, and the several feedback sessions were always insightful, which made executing and writing this thesis much less difficult. He was consistently accessible via various channels, and I could always seek his advice whenever any issues arose. Furthermore, I would also like to thank the staff who were active in the Studios for their cooperation in the laboratory. Especially Eline Taillieu, who helped me with various small appliance problems and other things like the positioning of certain equipment or reagents in the laboratory.

Lastly, I would like to thank my friends and family for not only supporting me through this thesis but also through the past four years.

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ABSTRACT

This study explored different technologies to increase protein extraction efficiency from yellow pea hulls. Pea hulls are an underutilized by-product of the food industry, and their valorization could therefore be economically and ecologically beneficial. The different applied technologies include ultrasound, enzymatic, a combination of the two, and high-pressure treatments.

Four ultrasound treatments with energy levels of 10 000 J, 50 000 J, 100 000 J, and 200 000 J were applied during aqueous extraction at pH 9. Enzymatic pretreatments using cellulase, pectinase, and their combination (0.1% w/v each) were conducted at 50°C and pH 5 for four hours, followed by alkaline extraction. The combined enzymatic-ultrasound treatment involved enzyme incubation followed by ultrasound of 10 000 J at pH 9. High-pressure treatments at 20, 40, and 60 bar for one hour at 20°C, followed by alkaline extraction were also explored.

The ultrasound-assisted extraction doubled crude protein yield compared to conventional extraction (approx. 20% vs. 40%). However, increasing sonication energy levels did not improve yields. Neither the enzyme treatments, the enzyme-ultrasound combinations, nor the high-pressure treatments impacted the protein yield, rendering them ineffective. Control samples from the enzyme treatments showed promising yields compared to conventional extraction (approx. 20% vs. 40%), suggesting that soaking pea hulls in mild acidic, warm conditions aids protein extraction. This soaking treatment may therefore be explored in further research.

Overall, while ultrasound is a promising method, other (pre)treatments require optimization or reevaluation for effectiveness. Generally, more insight into the protein fractions in pea hulls is necessary to enable a more targeted approach to improve extractability.

In dit onderzoek werden verschillende behandelingen om de efficiëntie van eiwitextractie uit gele erwtenhulzen te vergroten, onderzocht. Erwtenhulzen zijn een onderbenut bijproduct van de voedingsindustrie, de valorisatie hiervan kan daarom economisch en ecologisch gunstig zijn. De verschillende toegepaste behandelingen omvatten ultrasone, enzymatische, een combinatie van de twee en hogedrukbehandelingen.
Vier ultrasone behandelingen met energieniveaus van 10 000 J, 50 000 J, 100 000 J en 200 000 J werden toegepast tijdens alkalische extractie bij pH 9. Enzymatische voorbehandelingen met cellulase, pectinase en hun combinatie (elk 0,1% w/v) werden uitgevoerd bij 50°C en pH 5 gedurende vier uur, gevolgd door alkalische extractie. De gecombineerde enzymatisch-ultrasone behandeling omvatte enzymincubatie gevolgd door een ultrasone behandeling van 10 000 J bij pH 9. Hogedrukbehandelingen bij 20, 40 en 60 bar gedurende een uur bij 20°C, gevolgd door alkalische extractie, werden ook onderzocht.
De ultrasoon-geassisteerde extractie verdubbelde de opbrengst van ruw eiwit in vergelijking met de conventionele extractie (ca. 20% vs. 40%). Verhoging van de sonificatie-energieniveaus leidde echter niet tot hogere opbrengsten. De enzymatische, de combinatie van enzymen en ultrasound, en de hogedrukbehandelingen hadden geen invloed op de eiwitopbrengst en bleken dus niet effectief. Controlestalen van de enzymatische behandelingen toonden veelbelovende opbrengsten in vergelijking met conventionele extractie (ca. 20% vs. 40%), wat suggereert dat het weken van erwtenhulzen in mild zure, warme omstandigheden de eiwitextractie bevordert. Deze voorweekbehandeling zou kunnen worden onderzocht in verder onderzoek.
Over het algemeen is ultrasound een veelbelovende methode, maar andere (voor)behandelingen vereisen optimalisatie of herbeoordeling van de effectiviteit. Algemeen is er meer inzicht nodig in de aanwezige eiwitfracties in erwtenhulzen om een gerichtere aanpak mogelijk te maken, en zo de extractie-efficiëntie te verbeteren.

1. INTRODUCTION

There is a rise in the popularity of plant-based proteins. This is because animal proteins have an increasing negative connotation due to concerns about the ethics of livestock farming, the impact on the environment, and the perceived health concerns associated with the consumption of animal-based products. For instance, consumers show a concern about the high cholesterol levels in animal-based products. However, reducing the intake of animal products, which leads to a decrease in animal-derived proteins, requires proper replacement with alternative protein sources. This is because the human body relies on exogenous protein sources for amino acids which are needed for many physiological functions (Aimutis, 2022).

A shift towards more plant-based proteins also helps to combat the problems of food affordability and protein-energy malnutrition. By 2050, the world population is likely to exceed 9 billion. To meet the increasing food and feed demand, it is necessary to explore new protein sources (Langyan et al., 2022). Plant protein has the benefit of being efficient to produce: to produce 1 kg of animal protein, approximately 6 kg of plant protein is necessary (Daba & Morris, 2022).

The global meat alternatives market had a value of US$4.1 billion in 2017 and is projected to reach US$8.1 billion by 2026. The top 4 plant proteins used in such meat analogs are pea, soybean, wheat, and rice protein (Daba & Morris, 2022; Vatansever et al., 2020). Since the rise in interest towards plant-based proteins, however, a lot of different protein sources have also been explored and studied. These include more the more traditional protein sources like beans and peas, but also new sources such as protein from algae and more unconventional sources like by-products from the agro-industry (Langyan et al., 2022). This study will focus on pea hulls, a by-product of the processing process of peas.

2. LITERATURE REVIEW

2.1 Yellow pea

The interest in yellow peas (*Pisum sativum*) as a raw material for food applications is increasing. This is partly because of their favorable effects on the environment, which include nitrogen fixation and high output yields in regions with temperate climates (Millar et al., 2019). Additionally, their appeal includes lower allergenicity compared to soybeans and a non-GMO status (Stone et al., 2015). The yellow pea is a leguminous plant that is solely harvested for its seeds. These seeds are also called pulses and in contrast with other legumes like soybeans, they are characterized by a low lipid content. The proximate composition of yellow pea flour is 22.33 +/- 0.05% dry matter (DM) protein, 3.52 +/- 0.80% DM ash, 1.40 +/- 0.04% DM lipid and 14.84 +/- 0.93% DM total dietary fiber (Millar et al., 2019).

Due to its high protein content, the yellow pea could be a suitable alternative protein source. The economic value of pea protein was US$32 million in 2017 and is projected to hit US$176 million by 2025 (Daba & Morris, 2022). However, although the protein content in pulses may be high, the protein quality is considered poor compared to animal-derived protein. Pulses have a higher level of poorly digestible proteins and a high content of anti-nutritional factors including enzyme inhibitors and tannins. To solve this bioavailability issue, different processing methods can be employed (Millar et al., 2019). The pulse proteins also exhibit an imbalance of essential amino acids compared to animal proteins. The limiting amino acids in pulse protein are the sulfur-containing amino acids, methionine, and cysteine (Millar et al., 2019).

2.1.1 Proteins in yellow pea

The nutritional value and functional properties of pea protein are dependent on the quality and quantity of the protein. These two factors are affected by genetic factors, growth environment, and processing conditions. The primary components of pea protein are globulins and albumins. Respectively they account for 55-65% and 18-25% of the total protein mass. The globulins are composed of legumin, vicilin, and convicilin (Daba & Morris, 2022). However, the composition of the extracted protein depends largely on the extraction procedure. Alkaline extraction followed by isoelectric precipitation at pH 4.5 leads to a product that mainly consists of globulins. The albumins are lost during the precipitation step due to a difference in their isoelectric point (Tanger et al., 2020).

Legumin is a hexameric protein with a total molecular weight of 360 kDa. The protein comprises six subunits, each weighing 60 kDa. Each subunit consists of two components, an acidic part of 40 kDa and an alkaline portion of 20 kDa, linked together with a disulfide bridge. Vicilin is a trimeric protein with a molecular weight of 150 kDa, comprising three subunits, each weighing 50 kDa. Vicilin is less hydrophobic than legumin, which makes it more soluble in water. Convicilin is a tetrameric protein with a total weight of 280 kDa, consisting of four subunits, each weighing 70 kDa. Both vicilin and convicilin do not contain any disulfide bonds (Tanger et al., 2020). Pea

globulins tend to be high in arginine, phenylalanine, leucine, and isoleucine, while pea albumins are higher in tryptophan, lysine, and threonine (Stone et al., 2015).

The extraction of these proteins can be challenging since plant proteins are typically known for their limited solubility. The thermal denaturation occurring in potential industrial processing steps can further contribute to an even lower solubility. Besides that, the extraction of the soluble proteins is limited by the presence of vacuoles and rigid cell walls. Hence, the choice of extraction method is very important (Del Mar Contreras et al., 2019). There are many different options, such as salt extraction or micellar extraction, but in this review, there is a focus on the alkaline extraction of the proteins from biomass.

2.1.2 Pea hulls

Currently, peas are mainly consumed dehulled. A by-product of the processing of peas is therefore the pea hull. They account for approximately 7.2 - 12% DM of the peas and are mostly used in the feed industry (Ali-Khan, 1993; Guo et al., 2021). The hulls have a high content of dietary fiber and bioactive substances including polyphenols (Guo et al., 2021). Pea hull fiber is recognized as a dietary fiber and is primarily used to enhance the dietary fiber content of foods without significantly altering the fundamental properties of the product (Ratnayake & Naguleswaran, 2022).

The pea hull fiber content is estimated at 88.9% DM, the protein content at 5.2% DM, the starch content at 2.6% DM, and the ash content at 3.3% DM. The hull fibers contain various levels of cellulose, hemicellulose, and xylose rich pectin (Ratnayake & Naguleswaran, 2022). Gutöhrlein et al. (2020) executed a dry milling of pea hulls, followed by sieving. In such a manner, a fine (50 – 100 µm) and a coarse (250 – 350 µm) fraction were obtained. The fine fraction consisted of 65.6% DM total dietary fiber, 7.3% DM starch, and 12.5% DM protein. The coarse fraction consisted of 84.2% DM total dietary fiber, 0.4% DM starch, and 3.5% DM protein. The missing percentages of these fractions are either ash, or undefined.

Reinkensmeier et al. (2015) did a characterization of individual proteins in pea hull flour. An alkaline extraction, followed by an isoelectric precipitation, was used to isolate the protein from the pea hulls. For the characterization, SDS-PAGE was utilized. The results showed that the composition of the isolated protein fraction depends on the extraction pH. At pH 10.68 the protein isolate was largely composed of a high molecular fraction of α-legumin and of β-legumin. The smaller fractions include vicilin, albumin, and a low molecular fraction.
Valorization of pea hulls is possible by recovering and valorizing the protein fraction (Prandi et al., 2021). This has many advantages such as a reduction of environmental impact, the possibility to generate a more competitive agroindustry, and obtaining an additional economic benefit (Del Mar Contreras et al., 2019).

The cell walls (Figure 1) of the pea hull cells are a strong and compact structure of polysaccharides (i.e. cellulose, hemicellulose, and pectin). The cellulose and the hemicellulose interact with each other by crosslinking and electrostatic interactions. The polysaccharide networks are embedded in

a pectin matrix. Cellulose exhibits a multi-scale structure characterized by a highly organized arrangement of fibrillar components, including elementary fibrils, microfibrils, and macrofibrils (Morales-Medina et al., 2020). The cell wall provides a natural resistance and thus is one of the reasons for a low protein extractability. Some proteins are also embedded in the lignocellulosic matrix so a good extraction method is needed (Karabulut et al., 2023). This suggests that protein extraction from pea hulls is not easy. Technologies like ultrasound-, high pressure- or enzyme-assisted extraction may be used to aid the extraction. They are explored later in this review.

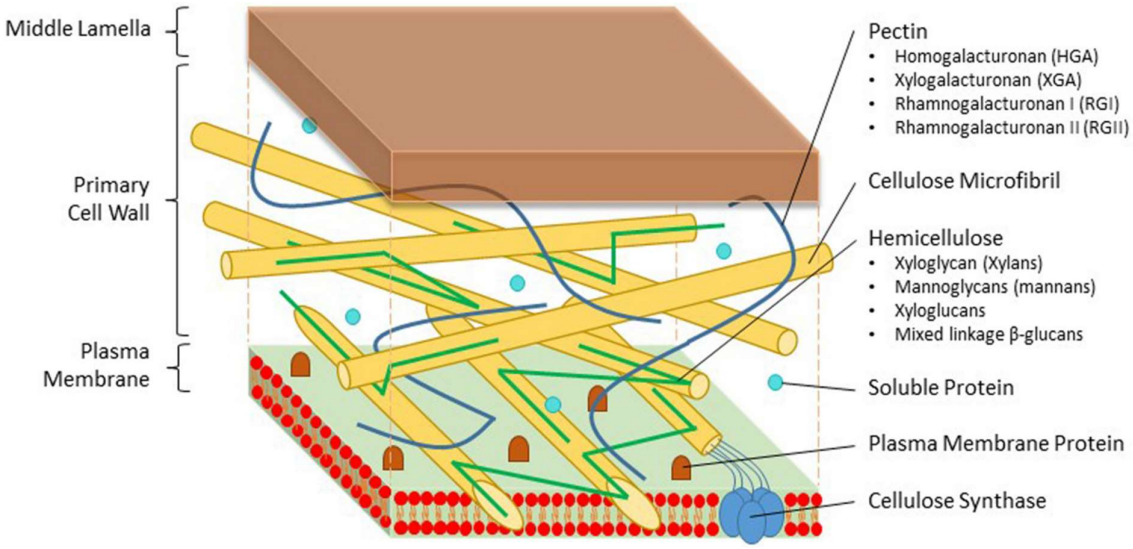

Figure 1: Cell wall diagram from a plant cell (retrieved from Loix et al.,2017)

2.2 Alkaline extraction of protein from biomass

A typical aqueous protein isolation process has two steps: a solubilization, which includes separation of the soluble residues via centrifugation, and an isoelectric precipitation. In the solubilization step, the proteins in the biomass are extracted using an alkaline water solution. The process is aided by shaking or stirring the sample. After separation via centrifugation, a precipitation step is performed. The proteins are made insoluble anew, making the separation from the water mixture possible, again, via centrifugation (Tanger et al., 2020).

The solubilization/protein-extraction step is under alkaline conditions. These alkaline conditions ionize the neutral and acidic amino acids, increasing protein solubility. Strong alkaline treatment of plant materials can lead to the unfolding of polypeptide chains due to the breakage of the disulfide cross-linking. Such conditions can also disrupt the cell wall surface, thereby diminishing its role as a structural barrier within the material matrix. Consequently, protein denaturation and cell wall disruption in a strong alkaline environment facilitate the diffusion of protein molecules into the surrounding aqueous extraction medium (Del Mar Contreras et al., 2019; Kamani et al., 2023). However, when the aim is to extract functional protein and thus avoid denaturation, milder alkaline conditions should be used (Jahan et al., 2022).

After centrifugation, the proteins are separated from the supernatant by adjusting the pH close to the isoelectric point of the target proteins. At the isoelectric point, the net charge of the protein molecules is zero. There is therefore no electrostatic repulsion, allowing more protein-protein interactions to occur. There are also fewer interactions between the protein molecules and water. Because of these conditions, it is favorable for the protein molecules to aggregate and precipitate (Kamani et al., 2023; Pelegrine & Gasparetto, 2005).

The efficiency of this isolation method is often not optimal, as proteins are lost in both the solubilization step and the precipitation step. According to the findings of Tanger et al. (2020), who investigated the extraction of protein from peas, the loss in the solubilization step is around 31.3% and the loss in the precipitation step is around 23.9%. Moreover, the protein yield is not the only factor that is affected by the isolation process, mainly the precipitation step also has a big impact on the protein profile of the final product. The authors concluded that albumins are mostly lost in the precipitation step. This is due to the different isoelectric points of both globulins (pH = 4.5) and albumins (pH = 6).

The alkaline extraction method described here is also influenced by a number of factors, including (among other factors) the pH, temperature, and biomass particle size used in the process (Kamani et al., 2023). These factors are discussed in the subsequent paragraphs.

2.2.1 pH

The pH has an important impact on the protein solubility. In the literature on legumes, protein solubilization is generally done at a pH of approximately 9.5 (Boye et al., 2010; Schmidt et al., 2022). However, Reinkensmeier et al. (2015) did a study on the protein solubility of specifically pea hulls at different pH levels. The samples were mixed with water with an adjusted pH level (by adding HCl or NaOH). Subsequently, the protein yield was determined. The pea hull extraction with water at pH 12 led to the highest yield. It is important to note that the pH of the mixtures was not re-adjusted after the pea hulls were added. So, the actual pH of the mixture, after the proteins were able to unfold their buffering potential, was 10.68 ± 0.03. The protein yield at pH 12 was still only approximately 25%. The authors therefore speculated that the majority of the protein in the pea hulls may include largely membrane-bound or highly hydrophobic proteins, which are not easily extracted under alkaline extraction.
A higher pH level may be advantageous to the protein solubility, but it can also cause undesirable changes in the protein molecule. At stronger alkaline conditions, the functional properties of the proteins are affected due to protein denaturation. A higher yield can therefore be at the expense of the functional properties of the end product (Jahan et al., 2022). Such conditions may also lead to reduced protein digestibility, thereby lowering the nutritional quality of the extracted protein products (Kamani et al., 2023).

2.2.2 Temperature

Increasing the temperature increases the protein yield until an optimal level. The optimum temperature that gives a maximum yield is usually between 30 and 50°C (Jahan et al., 2022). This

increase can be attributed to the dual effect on both the solute and solvent. A temperature increase leads to an increase in the desorption property and solubility of the solute in the solvent and it decreases the viscosity of the solvent which leads to an increased diffusivity of the solvent in the tissue matrix (Kumar et al., 2021).

The maximum yield at the optimal temperature is followed by a decrease in protein yield (Jahan et al., 2022). This can be ascribed to the effect of the temperature on the protein molecule itself. When the temperature is raised high enough for a given time, protein denaturation occurs. The temperature affects the noncovalent bonds involved in the molecule, e.g. hydrogen, hydrophobic and electrostatic bonds. These bonds are involved in the stabilization of the secondary and tertiary structure. When these structures unfold, the hydrophobic groups become exposed and they reduce water binding capacity. The hydrophobic interactions lead to aggregation and precipitation. Denaturation therefore generally decreases protein solubility compared to native proteins (Pelegrine & Gasparetto, 2005).

2.2.3 Biomass particle size

Biomass particle size also plays an important in the achievable protein extraction yield. Reducing the particle size enhances the surface area. This results in an elevated diffusion rate, making a smaller particle size preferable for enhanced protein extraction. Similar results were also obtained under ultrasound-assisted extraction (Jahan et al., 2022).
An option to reduce the particle size is microfluidization. Morales-Medina et al. (2020) did a study on the impact of this technique on the microstructure of pea hull fiber. Microfluidization is a high-pressure homogenization technique during which a pressurized suspension is forced through a microchannel (diameter < 400 µm). The technique results in the disruption of particles. They observed that the organized structure of cellulose largely disappears when the particle size decreases. This leads to interfibrillar voids and the release of soluble fiber.

2.3 Ultrasound-assisted protein extraction

Ultrasound (US) treatment is gaining popularity for solid-liquid extraction because of its notable advantages, including high reproducibility, shorter processing time, convenience, and reduced solvent usage (Bernardi et al., 2020). The treatment is typically used as a pretreatment at the solubilization step to improve the extractability of the protein (Del Mar Contreras et al., 2019).

Before discussing the impact on the protein extraction associated with ultrasound treatments, it is important to understand the fundamental nature of ultrasound waves. Ultrasound refers to sound waves or acoustic waves with frequencies above 20,000 Hz. The most common form of an acoustic wave is a longitudinal compressional wave, where the particles are displaced parallel to the direction of the motion of the wave. However, the particles are only displaced locally, i.e. they oscillate. This means there is no net flow of the fluid wherein the wave travels. Because of this displacement in particles, there are temporary regions of high density and low density. These regions correspond respectively with a high pressure (compression) and a low pressure (rarefaction) (Leighton, 2007).

The ultrasound waves create an acoustic cavitation within the extraction solvent. Ultrasound treatment enables the growth of pre-existing gas nuclei into bubbles that oscillate and subsequently collapse. The growth occurs in the rarefaction regions and the collapse in the compression regions (Figure 2). These bubbles are often accompanied by the formation of several physical and chemical effects, that can alter the matrix. The collapse of the bubbles results in micro-jetting, shockwaves, and local hotspots. This generates surface peeling, erosion, and particle breakdown. In this way, the cavitation can alter the cellular structure and elevate matrix porosity. Consequently, solvent penetration and mass transfer are enhanced. This process facilitates the release of extractable compounds like protein molecules. The chemical effects include the formation of reactive radicals (Del Mar Contreras et al., 2019; Kamani et al., 2023; Mondal et al., 2021).

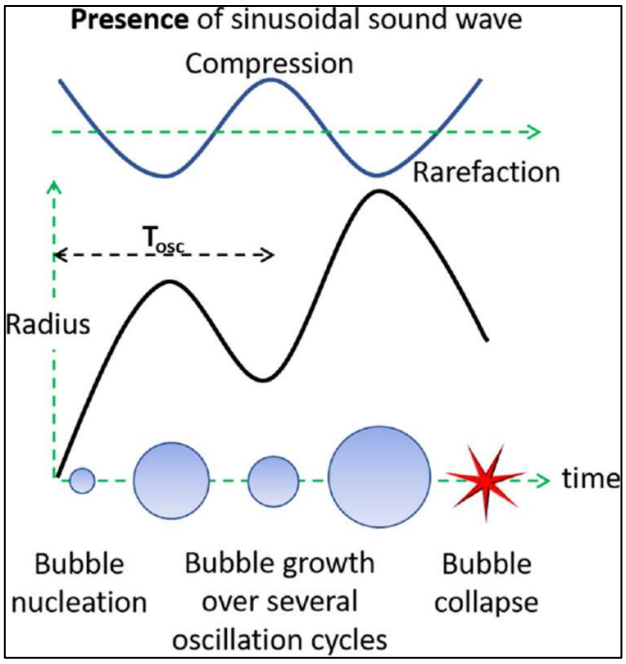

Figure 2: Formation of bubbles which leads to acoustic cavitation (retrieved from Mondal et al., 2021)

Sonication can not only affect the matrix, but also the structural characteristics of the protein molecules. They can undergo unfolding, denaturation, and reaggregation when exposed to ultrasonic cavitation. The breakdown of hydrophobic and hydrogen bonds results in conformational changes (i.e., secondary and tertiary structural changes), which can subsequently alter their techno-functional and nutritional properties in food systems. High-intensity ultrasound treatment can even break down proteins into smaller peptides, and therefore cause alterations in the primary structure (Ampofo & Ngadi, 2022). The changed protein conformation can also cause changes in their extractability (Hadidi et al., 2023).

The utilization of ultrasound in the food industry can be achieved by two approaches: by using a sonotrode or an ultrasonic water bath. In this study, a sonotrode will be utilized. The sonotrode

consists of a generator component that produces an alternating electric current with a specific ultrasound frequency. The transducer and amplifier components convert the generated current to mechanical forces and sound waves respectively. The sonotrode tip needs to be submersed into the extraction medium containing the plant substrate. Optimally, the tip should be submerged into the center of the sonoreactor, with a depth of approximately 2-3 cm; an inappropriate submersion of the tip can result in unfavorable consequences such as foaming and uneven distribution of acoustic energy and thus the cavitational effects. For a uniform distribution of acoustic energy, the sonoreactor should be characterized by a narrow, flat-bottomed, cylindrical shape (Ampofo & Ngadi, 2022). In the following paragraphs, the different parameters of this ultrasonic treatment will be discussed.

2.3.1 Frequency

Ultrasound treatments can be categorized by their frequency range. They can be classified into high-frequency low-intensity (MHz range) and low-frequency high-intensity ultrasound (kHz range).

A lower frequency is reported to have a greater effect on the protein extraction yield than a higher frequency. In a study on protein extraction from rice bran, the recovery was significantly higher for a treatment at 38 kHz than a treatment at 80 kHz (Bernardi et al., 2020). This could be due to a greater cavitation at lower frequencies. The generated bubbles are larger, so their collapse results in stronger shock waves. Cavitation bubble formation in higher frequencies is much more difficult because of the shorter compression and rarefaction cycles. The intensity of the waves cannot be increased indefinitely as the bubbles will become too large and they may not have enough time to collapse adequately in the compression phase of the ultrasonic wave. The inadequate collapse can limit the effectiveness of the ultrasound wave. A low-frequency, high-intensity ultrasound also generates strong shear and mechanical forces, which is desirable for the destruction of biomass (Bernardi et al., 2020; Kumar et al., 2021).

2.3.2 Ultrasonic power

The power delivered during an ultrasound treatment can also be expressed as an amplitude percentage in the range of 0 to 100% (with 100% indicating the rated power of the equipment), or as the power dissipated per unit volume of the extraction medium (Kumar et al., 2021).

An increase in power density increases the yield. One explanation for this increase is that the size of cavitation bubbles is proportional to the power of the ultrasonic wave. As the bubble size increases, the intensity of the implosion's impact also escalates. The mechanical vibrations of the probe also result in an increased surface area between the solid and solvent, which increases solvent penetration (Kumar et al., 2021). However, when the ultrasonic power exceeds a certain threshold, the protein solubility, and thus the yield, can start to decrease due to the hydrolysis and aggregation of the proteins (Xu et al., 2017). Excessive power levels can also result in too many bubble formations, which decreases the cavitation effect. This decrease can be attributed to an increased number of inter-bubble collisions, deformations, and non-spherical collapses which

decrease the impact of the bubble implosion. In addition to the lower impact, there is also the saturation effect; this is a reduced transmission of energy because of the layer of cavitation bubbles that assembles around the probe tip (Kumar et al., 2021).

2.3.3 Duty cycle of ultrasonication

A duty cycle is the ratio of pulse duration and cycle time of ultrasonic waves, expressed in a percentage. Pulse duration is the time when the ultrasonic transducer is turned 'on' and a pulse interval refers to the time for which the transducer is turned 'off'. The cycle time is the sum of the pulse duration and the pulse interval. A duty cycle helps to eliminate the influence of the temperature on the results, which leads to more accurate conclusions. An ultrasound operated in pulse mode decreases the number of bubbles but increases the intensity of the bubble implosion. At a high-duty cycle (50-70%) the cavitation effect decreases because of the previously discussed saturation effect and the inter-bubble collisions. The temperature also undergoes a more substantial increase, which can lead to protein denaturation, loss of solubility, and aggregation (Kumar et al., 2021; Li et al., 2021).

2.3.4 Extraction time

The ultrasonic time highly depends on the intensity of the power applied and the frequency generated by the ultrasonic system. For protein extraction from plant biomass, high power/short time sonication is preferred over low intensity/long time sonication. The high power/short-time sonication is less expensive and has a limited effect on protein denaturation and loss of functional properties. The low-intensity/long-time method has been reported to induce a higher temperature rise, resulting in protein denaturation. Besides the effect of the temperature, ultrasonic exposure can also damage the solute structurally and consequently reduce the extraction yield. However, within the high power/ short time category, a longer sonication time does initially result in a higher yield, which subsequently falls with the following time increments. The effect is similar to the effect of an increase in power and temperature. Upon the initial increase in time, the cavitation effect of the ultrasound increases the swelling, hydration, fragmentation, and pore development of the plant tissue matrix. All these elements facilitate the exposure of the solute to the extraction medium and thereby help their release into the solvent, resulting in an initially higher yield (Ampofo & Ngadi, 2022; Kumar et al., 2021).

2.3.5 Solid-liquid ratio

The solid-liquid ratio plays a crucial role in ultrasound-assisted protein extraction. In the development of an ultrasound-assisted extraction process, a broad range of solid-liquid ratios should be explored. In a study on the ultrasound-assisted protein extraction from wampee seeds by Jahan et al. (2022), an increasing solid-liquid ratio within the range of 1:20 to 1:30 led to an augmentation in protein yield. Nevertheless, a subsequent rise in the solid-liquid ratio (1:30 to 1:40) resulted in a decline in protein yield. This could be due to the diminishing significance of the ultrasonic energy density per unit volume (Jahan et al., 2022). The rise in the protein yield at a lower solid-to-liquid ratio could be because of the changed viscosity of the solution. At a high ratio,

the viscosity is also high, which poses more difficulties for the cavitation effect as the bubbles have to overcome a stronger cohesive force to grow in the viscous solution. A lower ratio also increases diffusivity due to a higher concentration difference in the solute (Kumar et al., 2021).

2.3.6 Temperature

The temperature should be slightly lower than the optimal extraction temperature, due to the rise in product temperature caused by the cavitation during the extraction period (Jahan et al., 2022). This rise is due to, when the bubbles collapse, the gasses inside are compressed adiabatically to very small volumes over a very short time. As a result, the gasses inside the bubble reach very high temperatures and pressures (Mondal et al., 2021). However, not all studies show a significant effect of the temperature on the protein yield, such as a study on rice bran by Bernardi et al. (2020). The study found that the temperature (within the range of 25-45°C) had no significant effect on the yield.

2.4 Enzyme-assisted protein extraction

Enzyme-assisted extraction is another approach to improve the extraction of protein from plant matrices. An enzyme pretreatment can be especially useful when the plant proteins are covalently or physically bound to other components of the plant tissue. The enzymes are either added before or during the alkaline treatment. If the enzymes are added before the treatment, the pH of the solution can be adjusted to the optimum value for the enzymes used. If the enzymes are added during the treatment, they should be capable of operating under these alkaline conditions (Hadidi et al., 2023).

Enzymes are used to either alter the matrix or the protein itself. Carbohydrases and proteases are the two types of enzymes used in this regard (Kamani et al., 2023).
Examples of carbohydrases are pectinases, cellulases, and hemicellulases. They target the integrity of the cell wall through enzymatic degradation. By hydrolysis of the lignocellulosic fraction, the proteins can be liberated from the polysaccharide-cell wall matrix. The compromise of the cell wall's structural integrity also results in increased permeability, allowing proteins within the cell to more readily migrate into the surrounding solvent. In this way, the extraction efficiency and recovery are improved (Del Mar Contreras et al., 2019; Kamani et al., 2023; Perović et al., 2020).
Examples of proteases are alcalase, flavourzyme, and neutrase. Proteases have the ability to hydrolyze peptide bonds within protein molecules, leading to the production of peptides with a lower molecular weight. This can enhance the solubility and hereby increasing the extraction yield. Proteases also act on the protein-polysaccharide matrix, thus releasing the protein. The effectiveness of these types of enzymes depends on the primary sequence of the protein. While some of them, like subtilisin, have broad specificity; others cannot work well if it is not favored by the protein's primary sequence (Del Mar Contreras et al., 2019; Kamani et al., 2023; Navaf et al., 2023).

In addition to the efficiency and recovery advantage of enzyme-assisted protein extraction, the method can also be seen as an environmentally friendly process. This is attributed to the use of

naturally occurring enzymes and the absence of hazardous waste generation (Kamani et al., 2023). However, the feasibility of this process on a larger scale is entirely dependent on the commercial availability and the cost of the needed enzymes and whether or not it is possible to scale up the process (Del Mar Contreras et al., 2019; Ranjan et al., 2023). To combat this economic problem, immobilized enzymes in biorefinery processes may be used as a more cost-effective alternative compared to using enzyme preparations (Hadidi et al., 2023).

2.4.1 Enzymes

Due to the differences in the specific cell wall components and structures of each plant species, a well-considered choice of enzyme plays a major role in the protein yield (Kleekayai et al., 2023). As previously discussed, the cell wall of pea and pea hulls contain cellulose and pectin. Therefore, cellulase and pectinase are discussed in the following sections.

2.4.1.1 Cellulase

Cellulases are a group of enzymes capable of degrading cellulose. The enzymes can be produced using fungi or bacteria, via a fermentation process. Cellulases are majorly represented by endoglucanase, exoglucanase and β-glucosidase. Endoglucanase randomly cuts the internal bonds of the cellulose chains and so produces glucose molecules and oligosaccharides of various lengths. Exoglucanase breaks the internal β-1,4 glycosidic bonds and thereby forms cellobiose units, oligosaccharides, and non-reducing sugars. β-glucosidase acts on specific locations in β-D-glucose molecules, cutting them and yielding smaller fragments of D-glucose as a result. Both endo- and exoglucanase help with the primary hydrolysis of the solid cellulose surface, with the production of soluble sugars of up to six glucose units as a result (Figure 3) (Ranjan et al., 2023).

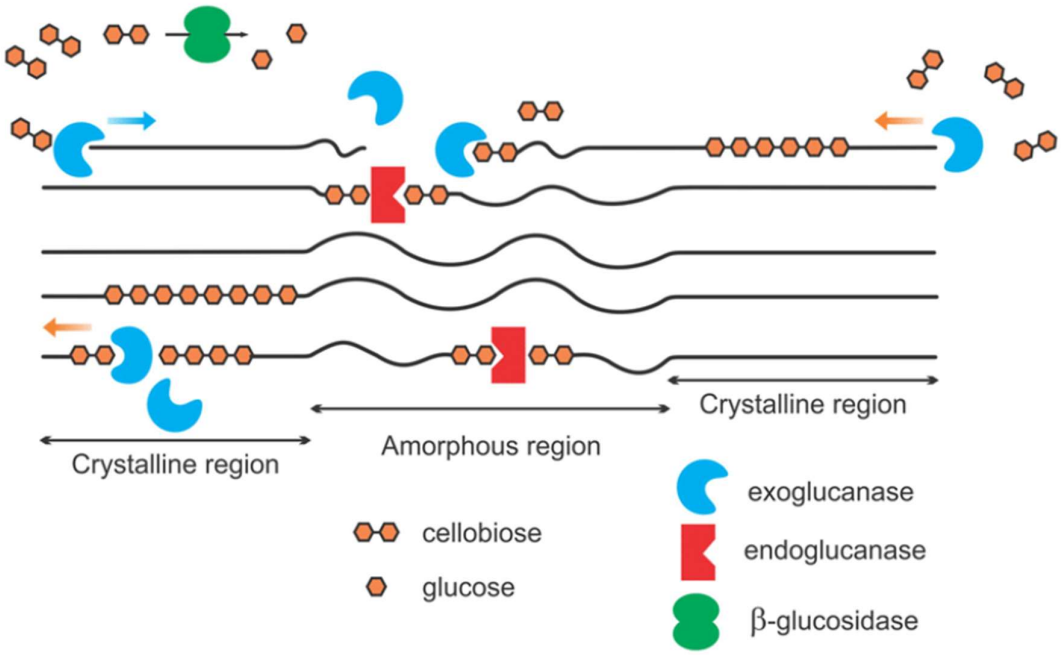

Figure 3: Schematic diagram of the enzymatic action of the different types of cellulose enzymes (retrieved from Nadeem et al.,2014)

2.4.1.2 Pectinase

Pectinases catalyze the degradation of pectic substances through depolymerization and de-esterification. Like cellulose, the enzyme group is mainly produced by microbes. Pectinases can be subdivided into protopectinases, depolymerases, and esterases. Protopectinases are hydrolases that cut down complex protopectin structures into pectin. Depolymerases depolymerize the pectinaceous matter into lower-chain compounds. The depolymerases can be further subdivided into hydrolases, which break the glycosidic bond of the pectic compounds, and lyases, which break the glycosidic bond at C4 and remove the hydrogen atom from C5, thereby forming an unsaturated product. Lastly, the esterases transform the pectin into pectic acid (Figure 4) (Patel et al., 2022). Since pectin is involved in the cross-linking of cellulose and hemicellulose fibers, pectinases could be able to help improve access of cellulases to their substrates (Kohli & Gupta, 2015).

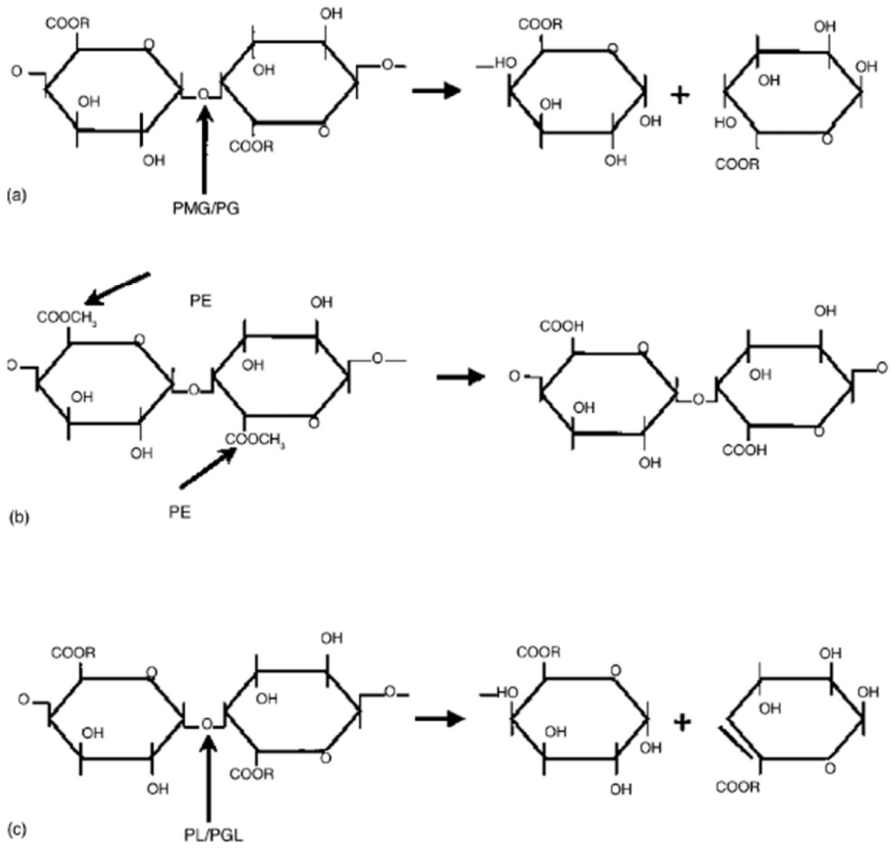

Figure 4: Mode of enzymatic action of different types of pectinases. (a) R = H for PG and CH_3 for PMG. (c) R = H for PGL and CH_3 for PL. PMG = polymethylgalacturonases; PG = polygalacturonases: both types of hydrolases. PE = pectinesterase. PL and PGL are both types of lyases (retrieved from Jayani et al., 2005)

2.4.2 Temperature

The optimal temperature for enzyme-assisted protein extraction is generally within the optimal range of the used enzyme (Kleekayai et al., 2023). Vergara-Barberán et al. (2015) did a study on enzyme-assisted protein extraction (with cellulase) from olive leaves. To determine the temperature with the maximum yield, various temperatures were tested from 25 – 65°C. It was

shown that a temperature increase had a significant, positive effect on the protein yield up to 55°C. Higher temperatures led to a slight decrease in yield, which can be explained by a possible thermal degradation of proteins. The relatively high optimal temperature is not only because of the increased enzyme activity but also because of the higher solubility of the protein molecules (Akyüz & Ersus, 2021). Enzyme mixtures with cellulase are mostly used in a range of 45 – 60°C and pectinase mixtures in a range of 45 – 50°C (Jung et al., 2006; Perović et al., 2020).

2.4.3 pH

The activity of an enzyme relies significantly on the conformation of its active and allosteric sites, which in turn is strongly impacted by the pH level. A shift in pH has the potential to modify the conformation and charge distribution of both the enzyme and the substrate. This could result in a decrease in enzyme activity and a reduction in the binding affinity between the enzyme and the substrate. Therefore, it is important to work in the optimal pH range of the used enzymes. This range is dependent on the enzyme used. Carbohydrases typically exhibit optimal pH levels within the mildly acidic pH range, whereas the optimum pH for the majority of commercially utilized food-grade proteases lies within the neutral to mildly alkaline pH range (Kleekayai et al., 2023). The pH also affects the substrate. In the case of protease, when the substrate is a protein molecule, the conformational structure and charge distribution of molecules can change. This affects the binding ability between the enzyme and the substrate, and thus affects the protein yield (Singh et al., 2019). Enzyme mixtures with cellulase are mostly used in a pH-range of 4 - 7 and pectinase mixtures in a pH-range of 4 – 4.5 (Jung et al., 2006; Perović et al., 2020).

2.4.4 Duration of hydrolysis

Protein yield reaches a maximum after a certain amount of incubation time with the enzyme. This could be due to the deactivation of the enzyme during long periods of incubation and because of higher levels of hydrolysis products present, which may act as enzyme inhibitors. A long incubation period at high temperatures can also promote microbial growth; which could be a concern during the manufacturing process. Shorter hydrolysis times can be achieved by using higher enzyme concentrations or with biomass pretreatment (Kleekayai et al., 2023). A study on rice bran suggests (with papain enzyme, a protease) an optimal period for enzymatic hydrolysis of 150 minutes. In these initial 150 minutes, the reaction rate was fast; subsequently, the hydrolyzing velocity decreased (Singh et al., 2019).

2.4.5 Enzyme concentration

It is well documented that an increasing enzyme concentration results in a greater protein yield (Kleekayai et al., 2023). For example, a study on protein extraction from olive leaves (cellulase-assisted) looked into the effect of the used enzyme concentration (Vergara-Barberán et al., 2015). Enzyme percentages varied from 0 to 8% (v/v). A peak in extraction yield was reached at 5%; the subsequent decline can be explained by the appearance of turbidity in the protein extracts (when using > 5% (v/v) enzyme) caused by the interaction of free enzymes and extracted protein molecules, which causes degradation. Another explanation is competitive inhibition, in which the

extracted protein molecules bind with the enzymes thereby preventing their activity, although there is no evidence of this mechanism (Vergara-Barberán et al., 2015). At a certain point, after excessive hydrolysis, the possibility of contact between the enzyme and substrate molecules is vastly reduced. Thus the rate of hydrolysis becomes static (Singh et al., 2019).

2.4.6 Particle size

A smaller particle size generally leads to a higher extraction yield due to increased surface accessibility by the enzymes. It additionally promotes the diffusion of intracellular components into the surrounding aqueous environment (Kleekayai et al., 2023).

2.4.7 Solid-liquid ratio

The substrate concentration used during the protein extraction determines the enzyme accessibility during the extraction process. The used concentrations are generally in the region of 5-15% (w/v). Working at a lower concentration would mean that further downstream processing is necessary to have a higher protein concentration. Working at a higher concentration can negatively impact the hydrolysis rate. Therefore, an optimum point needs to be determined to achieve the highest possible yield (Kleekayai et al., 2023). As observed in a study on sugar beet leaves; an optimum was reached at 0.036 g/ml (Akyüz & Ersus, 2021).

2.5 High-pressure assisted protein extraction

High-pressure processing can be used to facilitate protein extraction from plant sources due to the disruption of plant cells and protein aggregates. The method is an emerging green technology for all types of extractions (Mustafa & Turner, 2011).

When the pressure is increased to the intended level (100 – 800 MPa) from the atmospheric threshold, the variance in pressure between the interior and the exterior of the cell escalates (Hadidi et al., 2023; Navaf et al., 2023). Increased pressure causes cell damage and deformation, resulting in greater permeability of the cell membrane. This allows for improved penetration of solvents into the cells, thereby enhancing the release of proteins or other compounds of interest (Suchintita Das et al., 2023).

The set pressure is upheld for a certain period, ensuring an equilibrium is reached between the internal and external pressure of the cell. When the pressure is released, the built-up pressure in the cells goes back to the atmospheric pressure. This process involves cell expansion due to the trapped gas, leading to deformation. Accelerating the pressure release induces more pores within the cell structure, which increases the surface area of the plant material and ultimately results in a higher yield (Hadidi et al., 2023).

The high pressure does not only affect the plant matrix; it may also affect the proteins. High-pressure processing can induce protein denaturation and subsequent rearrangement of inter- and intramolecular interactions. New conformations are generated that may exhibit modified

functional properties (Hall & Moraru, 2021). Proteins undergo reversible folding/unfolding transitions when subjected to pressures of 200 – 1000 MPa (Hillson et al., 1999).

High-pressure assisted protein extraction is dependent on several variables, such as the solid-liquid ratio, the extraction pressure, and the duration of the process (Hadidi et al., 2023). The parameters used for the high-pressure extraction of proteins from *Arthrospira platensis* are pressures ranging from 100 to 600 MPa, extraction times from 0 to 10 minutes, and a processing temperature of 20°C (Giannoglou et al., 2022).

The high pressure used in this study on a laboratory scale will be 20 to 60 bar (2 to 6 MPa), lower-pressure applications like this have not been studied in the literature. No higher pressures could be achieved with the available equipment.

2.6 Comparison between the different extraction methods and the possibility of combining the methods

The different extraction methods each have advantages and disadvantages. Both enzyme-, US- and high-pressure assisted extraction methods generally result in higher yields than traditional alkaline extraction. Traditional extraction also has the disadvantage of lower quality protein yield, as during the extraction racemization and denaturation of the protein molecules can occur. But the non-traditional methods have a higher cost compared to the traditional alkaline extraction and may be less simple to run (Hadidi et al., 2023; Navaf et al., 2023). Table 1 provides a summary of the advantages and disadvantages of the different extraction techniques.

Karabulut et al. (2023) did a study on pea pods protein extraction and used different extraction methods to compare their yields. After alkaline extraction, the protein yield was only 16.2%. The enzyme-pretreated alkaline extraction (pectinase, Viscozyme®, and a combination of the two) provided significantly lower protein yields (8% - 9.1%) than the alkaline extraction on its own. This could be due to the different solid-to-liquid ratios used in the alkaline extraction (1:20) and the enzyme-assisted extraction (1:10). Since higher extraction rates are expected at greater dilutions, this result is not that surprising. Ultrasonic-assisted extraction (30% ultrasonic amplitude and 5 min of sonication), however, did lead to a higher extraction yield of 21.1%.
In a different study on protein extraction from mulberry leaves, the yields between the different extraction methods were also compared. In this study, both the cellulase-assisted (enzyme concentration of 0.66 g/l for 30 min) extraction and the US-assisted (240 W/l and 25 min of sonication) extraction had a higher yield than the traditional alkaline extraction. The US-assisted extraction had the highest yield of the three (Zhao et al., 2023).

Besides the protein yield, Karabulut et al. (2023) did an examination of the molecular weight distribution of the extracted pea pod proteins using different methods. Polypeptides greater than 50 kDa and legumin-β could not be detected regardless of the extraction method. The enzyme-assisted extraction resulted in molecules with a mean weight of 37 kDa, representing legumin-α subunits. The US-treated samples had a high amount of molecules with a lower molecular weight of 15 kDa, which the authors attributed to the cavitation effect on the protein structure.

A study on the extraction of protein from faba beans using several innovative technologies was done by Suchintita Das et al. (2023). High-pressure assisted extraction was performed at 300 and 600 MPa for 3 minutes; ultrasound-assisted protein extraction was performed at 20 kHz, 100% amplitude, and 57.58 W/m^2 and for the conventional method, the samples were stirred at 500 rpm for 18 hours. No significant differences were observed in either the protein content or the extraction yield between the methods.

The exploration of combined treatments, such as ultrasound-assisted enzymatic extraction and microwave-assisted enzymatic extraction, is increasing due to the higher yields that can be obtained. Reports suggest that the application of both ultrasound and enzymes enhances the extraction yields of various compounds, including arabinoxylan and polysaccharides. However, further studies are required to shed more light on the synergistic interactions between ultrasound technology and enzyme application. Though, the general hypothesis is that the cavitation effects of the ultrasound waves lead to better enzyme-substrate interactions, thus resulting in higher recovery rates (Görgüç et al., 2019).

As previously discussed, an ultrasound treatment can be used as a biomass pretreatment for the enzymatic-assisted alkaline extraction. The ultrasonic pretreatment changes the surface structure of the substrate, such as an increase in roughness or the production of pores and grooves. The US-treatment can also affect the size of the substrate and it can peel off targets bound to other substances. All this provides a larger surface area of the substrate. Because of this, the formation of an enzyme-substrate complex is easier, and as such the efficiency of the hydrolysis increases (Qian et al., 2023). The treatments could also be done in reverse, where the enzymatic treatment is a pretreatment to the US-treatment, or the two can be done simultaneously. There is an increase in enzyme activity under mild ultrasonication when the shear force, temperature, pressure, and production of radicals are limited by controlling the power and the sonication time (Khadhraoui et al., 2021).

Görgüç et al. (2019) studied the protein extraction from sesame bran by enzyme-assisted extraction, ultrasound-assisted extraction, and a combination of the two types of extraction at the same time. The highest protein yield (87.9%) was achieved through combined ultrasound-assisted enzymatic treatment (Alcalase with a concentration of 1.824 Anson unit/100 g; P = 759 W, T = 48°C and a processing time of 93 min), followed by enzymatic extraction using Alcalase (79.3%), (1.272 Anson unit/100g; pH = 11; T = 40°C and an incubation time of 65 min), ultrasound-assisted extraction (59.8%) (P = 682 W, T = 55°C and a sonication time of 65 min), and enzymatic extraction using Viscozyme L (41.7%)(91.2 Fungal beta-glucanase/100g; pH = 5.1; T = 48°C and an incubation time of 93 min).

Table 1: Comparison between different extraction techniques (based on Hadidi et al., 2023; Navaf et al., 2023).

Extraction technique	Advantages	Disadvantages	Scalability
Traditional alkaline process	- Simple extraction - Low cost	- Racemization - Long processing time - Denaturation - Low functional properties	Industrial scale
Ultrasound-assisted alkaline extraction	- Enhanced extraction yield - Reduced processing time - High extraction efficiency - Environmentally friendly - Low cost - Non-toxic	- High energy consumption - Denaturation - High capital investment	Pilot scale
Enzyme-assisted alkaline extraction	- Faster extraction - Low energy consumption - Environmentally friendly - High degree of selectivity and specificity	In long incubation: - Microbiological growth - High cost of processing - Product transformations	Laboratory scale
High-pressure assisted alkaline extraction	- Inactivation microorganisms - No toxicity - Uniform treatment - Low thermal degradation of protein	- Equipment cost - Formation of unwanted by-products - Denaturation	Industrial scale

3. CONTEXT AND AIMS OF THIS STUDY

This study aims to maximize the protein yield extracted from yellow pea hulls by exploring different strategies. The explored strategies are ultrasound, cell wall degrading enzymes, a combination of enzymes and ultrasound, and high-pressure treatments. Pea hulls are already used in the food industry, mostly as a dietary fiber but also as a source of phenolic and antioxidant components. Few studies focus on protein extraction and the exploration of different technologies to aid this extraction.

4. MATERIALS AND METHODS

4.1 Yellow pea hulls

The yellow pea hulls were obtained from Cosucra NV (Warcoing, Belgium). They were obtained after cleaning, destoning, and dehulling yellow peas. The hulls used for the experimental work have undergone a milling and thermal treatment.

4.2 Protein extraction

Protein isolation usually consists of two steps: a solubilization step and a precipitation step. In this study there is a focus on the protein extraction yield. Consequently, only protein solubilization is executed, and not protein precipitation.
Numerous samples were generated using various treatments. A clear overview of these samples is shown in Table 2. Each extraction procedure was conducted in triplicate. Preliminary experiments regarding overnight soaking combined with ultrasound-assisted extraction were conducted only once. Each procedure is described in more detail below.

Table 2: Overview of the extraction methods.

	Type of extraction method
Conventional	Conventional (= solely alkaline extraction)
Enzymatic	Control enzyme (= buffer without enzymes)
	Pectinase
	Cellulase
	Pectinase and cellulase
Ultrasound (US)	10 000 J
	50 000 J
	100 000 J
	200 000 J
Soak overnight (SO) + US	SO + 10 000 J
	SO + 50 000 J
	SO + 100 000 J
	SO + 200 000 J
High pressure	20 bar
	40 bar
	60 bar
Enzymatic + US	Control enzyme (= buffer without enzymes) + US
	Pectinase + US
	Cellulase +US
	Pectinase and cellulase + US

4.2.1 Conventional alkaline extraction

An extraction vessel was prepared with 1.25 grams of pea hull powder, and the exact weight was noted for future calculations. 50 ml of demineralized water was added, and the pH of the mixture was adjusted to 9 using 2 M NaOH. The sample was shaken at 150 rpm for two hours.

4.2.2 Enzyme-assisted extraction

An extraction vessel was prepared with 1.25 grams of pea hull powder, and the exact weight was noted for future calculations. 50 ml of acetate buffer solution (pH 5.0, 0.05 M) was added. The sample was then pre-incubated in a water bath at 50°C for 15 – 20 minutes.
Enzymes (50 µl; cellulase and/or pectinase) were added to achieve a concentration of 0.1% v/v. The cup was closed and turned to homogenize. Subsequently, the sample was incubated for four hours at 50°C with the shaker at approximately 150 rpm.
After incubation, the pretreatment was stopped by adjusting the pH to 9 with 2 M NaOH. The sample was put on a shaker for two hours at 150 rpm.

The enzymes used were Cellulase, specifically Celluclast® 1.5 L (Novozymes, Bagsvaerd, Denmark), and Pectinase, which was the endo-pectin lyase ROHAPECT® PTE100 (PF, AB Enzymes, Darmstadt, Germany).

To create a suitable control, the same procedure was followed without the addition of enzymes.

4.2.3 Ultrasound-assisted alkaline extraction

A 100 ml glass beaker was prepared with 1.25 grams of pea hull powder, and the exact weight was noted for future calculations. Subsequently, 50 ml of demineralized water was added, and the pH of the mixture was adjusted to 9 using 2 M NaOH.
The beaker was then placed in an ice bath beneath the ultrasonic probe. Carefully, the ultrasonic probe was lowered into the sample, ensuring it was centered properly.
Following this, the parameters of the device were adjusted: pulsing mode (c) was set to 50%, and power level (P) was set to 100 W. The duration of sonication depends on the applied energy level. In this study, energy levels of 10 000 J, 50 000 J, 100 000 J, and 200 000 J were utilized. The sonication process was then initiated.

For the soaked overnight samples, the 1.25 grams of pea hull powder was first soaked overnight in 50 ml demineralized water (pH 9) in an extraction recipient. Subsequently, the sample was transferred to a 100 ml glass beaker and the sonication was performed in the same way as described above.

4.2.4 High-pressure assisted alkaline extraction

An extraction vessel was prepared with 5 grams of pea hull powder, and the exact weight was noted for future calculations. Subsequently, 200 ml of demineralized water was added, and the pH was adjusted to 9 with 2 M NaOH.
The sample was then transferred into the high-pressure reactor, and the reactor was appropriately closed. Target pressure levels of 20, 40, or 60 bar were applied in this study, Table 3 gives the actual pressure levels achieved. The reactor remained under pressure for one hour, while a stirring rod continuously stirred the mixture at 100 rpm during the pressure treatment.
After the one-hour pressure treatment, the pressure was released, and the sample was transferred back to an extraction recipient. The sample was then shaken for one hour at 150 rpm.

Table 3: The actual pressures used when performing the high-pressure treatments.

Target pressure (bar)	Pressure utilized (bar)
20	22.2 ± 0.6
40	43.4 ± 1.0
60	62.4 ± 1.8

4.2.5 Combination of enzymatic and ultrasound-assisted alkaline extraction

An extraction vessel was prepared with 1.25 grams of pea hull powder, and the exact weight was noted for future calculations. Subsequently, 50 ml of 0.05 M acetate buffer solution was added to adjust the pH to 5. The sample underwent pre-incubation in a water bath at 50°C for 15 – 20 minutes.
Following this, enzymes (50 µl; cellulase and/or pectinase) were added to achieve a concentration of 0.1% v/v, and the cup was closed and turned to homogenize the sample. The sample was then incubated for four hours at 50°C with the shaker set to approximately 150 rpm.
After the incubation period, the pretreatment was halted by adjusting the pH to 9 with 2 M NaOH. The sample was transferred to a 100 ml glass beaker and placed in an ice bath beneath the ultrasound probe. The settings were adjusted to c = 50%, P = 100 W, and the applied energy level was set to 10 000 J. Carefully, the ultrasonic probe was lowered to ensure proper centering, and the sonication process was started.

To establish a suitable control, the same procedure was followed without the addition of enzymes.

After each extraction method, the sample was centrifuged at 4000 rpm for ten minutes at 20°C. Subsequently, the supernatant was separated via a filter, and the total weight was noted. The supernatant was then divided into different falcons: two with approximately 5 ml supernatant for crude protein determination, two with approximately 10 ml supernatant for true protein determination, and one with approximately 10 ml for dry matter (DM) determination. All the exact weights were recorded for future calculations, these weights will be referred to as ‘sample mass’.

4.3 Protein determination

For protein determination, the Kjeldahl method was employed. Initially, the nitrogen content was measured, and subsequently, the protein content could be estimated using a conversion factor. Typically, for most types of biomass, this factor is 6.25 (Del Mar Contreras et al., 2019).

The Kjeldahl method encompasses three phases: destruction, distillation, and titration.
In the destruction phase, the samples were quantitatively transferred into Kjeldahl tubes. To each tube, a Kjeldahl tablet, acting as a catalyst, was added (Kjeldahl tablets from Sigma-Aldrich, article n° 1.17958). Following this, 20 ml of sulfuric acid (95 – 97%) was added, and the tubes were placed into the destruction chamber. Heating ensued until complete destruction was achieved, indicated by the translucent green color of the samples. Typically, this phase requires one to two hours. Subsequent to cooling, 50 ml of demineralized water was added to further cool down the tubes. During this phase, nitrogen present in the sample is converted into NH_4^+ ($(NH_4)_2SO_4$), while the remaining carbon and hydrogen are converted into CO_2 and water (Altuntas & Hapoglu, 2019).

In the distillation phase, 25 ml of boric acid at a concentration of 10 g/l along with 3 drops of Tashiro's indicator, was prepared in an Erlenmeyer flask. Once the distillation unit was preheated, a destruction tube was connected, and the Erlenmeyer flask was positioned at the outlet. Sodium hydroxide (NaOH) was then added to the tube to neutralize it and convert any ammonium ions (NH_4^+) present into ammonia (NH_3). The mixture underwent distillation for 4 minutes. Consequently, the NH_3 released was absorbed into the boric acid solution, forming ammonium borate ($(NH_4)_3BO_3$). The pH change in the solution causes the color in the flask to transition from pink to light green (Altuntas & Hapoglu, 2019).

Finally, in the titration phase, the obtained Erlenmeyer flask was titrated with 0.025 M HCl solution until the pink color reappeared. The amount of HCl used was noted down.

With the HCl volume, the amount of nitrogen (in grams) in the sample can be calculated using formula 1.

$$N\ (g) = volume\ HCl\ * concentration\ HCl * molar\ mass\ nitrogen \quad (1)$$

The protein amount (in grams) can be calculated by the multiplication of the amount of nitrogen with a conversion factor (formula 2). This conversion factor is dependent on the type of product. In literature, a conversion factor of 5.36 is used for pea protein.
The aforementioned conversion factor is the average of three conversion factors proposed by different authors for pea protein. 5.24 is proposed by Sosulski and Imafidon (1990), 5.40 is proposed by Sarwar et al. (1983), and 5.44 is proposed by Mossé (1990). This data was grouped in an article on converting nitrogen into protein by Mariotti et al. (2008).

$$Protein\ (g) = N(g) * conversion\ factor \quad (2)$$

4.3.1 True protein determination

Because nitrogenous compounds in foodstuffs encompass more than just proteins or amino acids, determining the true protein (TP) value becomes essential. Other possible nitrogenous compounds include nucleic acids, amines, urea, ammonia, nitrates, nitrites, phospholipids, nitrogenous glycosides, etc. (Mariotti et al., 2008).

Unlike crude protein (CP), where the whole sample is transferred and thus all the nitrogen is measured, true protein only measures the nitrogen from proteins. To separate the proteins from the supernatant, 40% w/v trichloroacetic acid (TCA) precipitation was used.
The TCA was added in the falcons for TP (approximately 10 ml) in a 1:2 ratio, achieving a final TCA concentration of 20%. The sample was turned to homogenize and put in an ice bath for 1 hour, and subsequently centrifuged at 4000 rpm for ten minutes at 4°C. The supernatant and the pellet were separated, with the TP in the pellet. Consequently, the pellet can be quantitively transferred in a destruction tube for Kjeldahl analysis.

4.3.2 Protein yield calculations

To determine the protein content of the supernatant (g/g fresh weight (FW) extract), the calculated protein amount was divided by the sample mass. The sample mass was defined as the weight of the supernatant in the falcon dedicated to CP or TP determination (formula 3).

$$protein\ content\ (\frac{g}{g\ FW\ extract}) = \frac{protein}{sample\ mass} \quad (3)$$

For the protein yield, the total extracted protein (in grams) was first calculated by multiplying the protein content with the total sample mass, with the total sample mass being the total weight of the supernatant (formula 4).

$$extracted\ protein\ (g) = protein\ content * total\ sample\ mass \quad (4)$$

Two sequential formulas were applied to determine the protein amount (in grams) of a fresh sample.
Firstly, formula 5 was utilized to calculate the DM weight of the sample. This involved multiplying the sample weight (of the pea hull powder) by the dry matter percentage of the pea hull powder. Subsequently, formula 6 was applied. This formula involved multiplying the (true or crude) protein content of the pea hull powder by the DM weight of the sample.
These calculations relied on previously determined values: the DM percentage, TP content, and CP content which were established in a prior study. For the pea hull powder, the DM content was 93.96 grams per 100 grams of fresh weight; the TP content was 4.95 grams per 100 grams of DM weight, and the CP content was 5.52 grams per 100 grams of dry weight. Thus, the ratio of TP to CP content is 89.7%.

$$DM\ weight\ (g) = DM\ percentage * sample\ mass\ (pea\ hull\ powder) \quad (5)$$

$$protein\ in\ fresh\ sample\ (g) = protein\ content\ (pea\ hull\ powder) * DM\ weight \quad (6)$$

Finally, the protein yield can be calculated by dividing the extracted protein by the protein in the fresh sample (formula 7).

$$protein\ yield\ (\%) = \frac{extracted\ protein}{protein\ in\ fresh\ sample} * 100 \quad (7)$$

The ratio of TP content to CP content was calculated with formula 8.

$$content\ ratio\ (\%) = \frac{TP\ content}{CP\ content} * 100 \quad (8)$$

4.3.3 Dry matter determination

To determine the DM yield and the protein yield per DM content, the falcon of approximately 10 ml, intended for DM determination, was freeze-dried. The residue after freeze-drying was weighed.
To calculate the DM yield, the weight of the DM in the whole supernatant had to be calculated first (in grams). The measured weight after freeze-drying (FD) was multiplied by the total sample mass of the supernatant, this was then divided by the sample mass of the falcon that was freeze-dried (formula 9).

$$extract\ FD\ weight\ (g) = \frac{weight\ after\ FD * total\ sample\ mass}{sample\ mass} \quad (9)$$

Thereafter, the DM yield was calculated with formula 10.

$$DM\ yield\ (\%) = \frac{extract\ FD\ weight}{DM\ weight} * 100 \quad (10)$$

Lastly, the ratio of the extracted protein to the DM content of the supernatant can be determined with formula 11. This value is expressed as g/ 100g DM of the extract.

$$Amount\ of\ protein\ to\ the\ DM\ weight\ of\ the\ supernatant\ \left(\frac{g}{100g\ DM\ extract}\right) = \frac{extracted\ protein}{extract\ FD\ weight} * 100 \quad (11)$$

4.4 Microscopy

To get more insight into the structural changes that occur during the different extraction methods, the pellets of the samples were studied under a light microscope. The 10x ZEISS N-ACHROPLAN lens was used and pictures of the samples were taken with ZEN microscopy software from ZEISS. Following the capture of the images, a scale bar was added utilizing the software.

4.5 Statistical analysis

Comparisons between the different results were done by performing an (one-way) ANOVA analysis, followed by a Tukey-Kramer test as a post-hoc analysis in R, with the used significance level being 0.05.

5. RESULTS

The results of both the crude protein (CP) and true protein (TP) yield and the CP and TP content for all the executed treatments are shown in Appendix One. In this chapter, the obtained data are discussed, and statistical comparisons are made. Statistically significant differences ($p < 0.05$) are shown via Compact Letter Display. The results are discussed in the following order: first, the outcomes of ultrasound extraction, followed by enzyme-assisted extraction, high-pressure assisted extraction, and the extraction with combined enzymatic-ultrasound treatment. Subsequently, a comparison of the different control samples is made, the TP to CP content ratio is explored, and finally, the results of dry matter calculations and microscopic analysis are discussed.

5.1 Ultrasound-assisted alkaline extraction

The protein yield from the ultrasound-assisted extraction is shown in Figure 5. The CP yield is higher after the ultrasound treatment (all sonication levels). A significant difference was observed between the conventional sample and the following ultrasound samples: US 10 000 J, US 100 000 J, and US 200 000 J; but not between the conventional and US 50 000 J samples ($p = 0.015$). However, all the ultrasound treatments gave roughly the same outcome regardless of the sonication level, with no significant differences in CP yield across the various levels.
Never was there any significant difference observed between the TP yield of the different extraction methods. The difference may not be significant, but the TP yield approximately doubled after the different ultrasound treatments compared to the conventional treatment ($p = 0.478$).

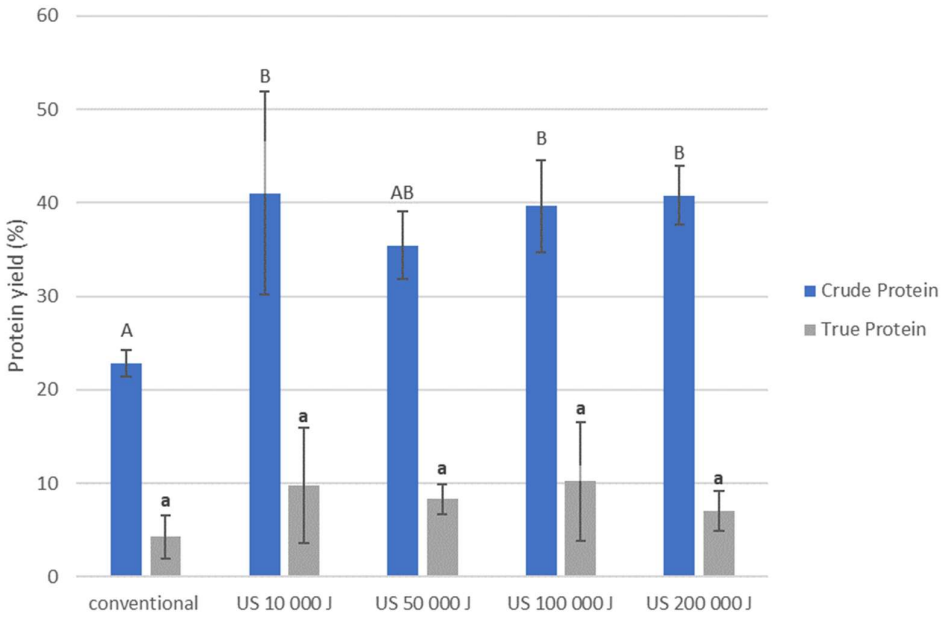

Figure 5: Protein yield after ultrasound (US)-assisted extraction. With 10 000 J, 50 000 J, 100 000 J, and 200 000 J indicating the energy level of the sonication. The conventional extraction sample serves as a control. Statistical significance is shown via Compact Letter Display (Capital letters for CP yield, lowercase letters for TP yield). The experiments were done in triplicate.

The protein yield from the samples that were soaked overnight (SO), followed by an ultrasound-assisted extraction is shown in Figure 6. This preliminary experiment was conducted only once; therefore, statistical analysis could not be performed. The CP yields are around 35% (except SO + 10 000 J) which is comparable to the yields after only the ultrasound-assisted extraction (Figure 5).

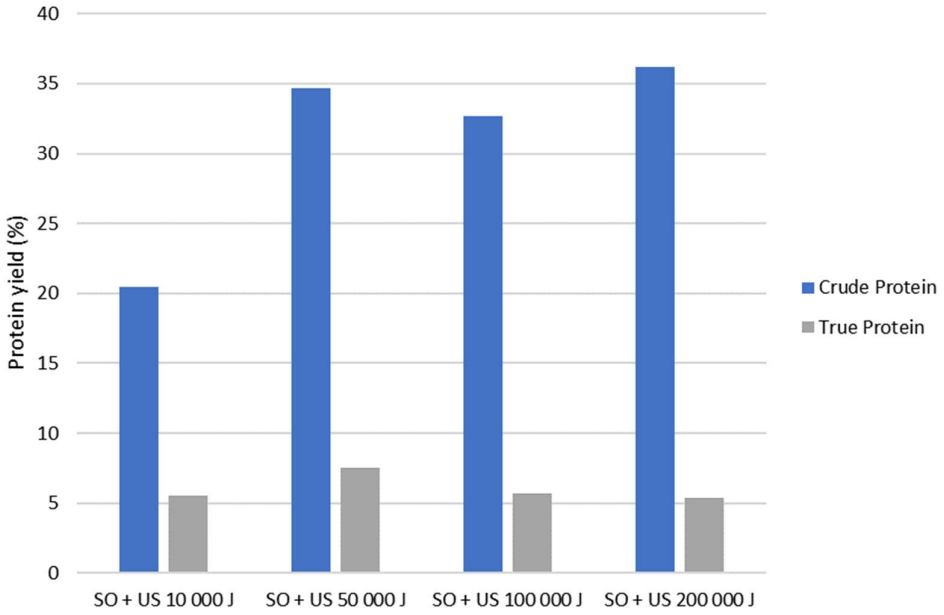

Figure 6: Protein yield after the sample was soaked overnight, followed by an ultrasound (US)-assisted extraction. With 10 000 J, 50 000 J, 100 000 J, and 200 000 J indicating the energy level of the sonication. And SO indication soaked overnight. the experiment was conducted in a singular.

5.2 Enzyme-assisted extraction

The protein yield from enzyme-assisted extractions is presented in Figure 7. There were no significant differences in CP yield across different enzyme pretreatments or in TP yield across the treatments, including the control (CP: p = 0.063; TP: p = 0.135). Although insignificant, the control has a higher CP yield by approximately 10% compared to different enzyme treatments.

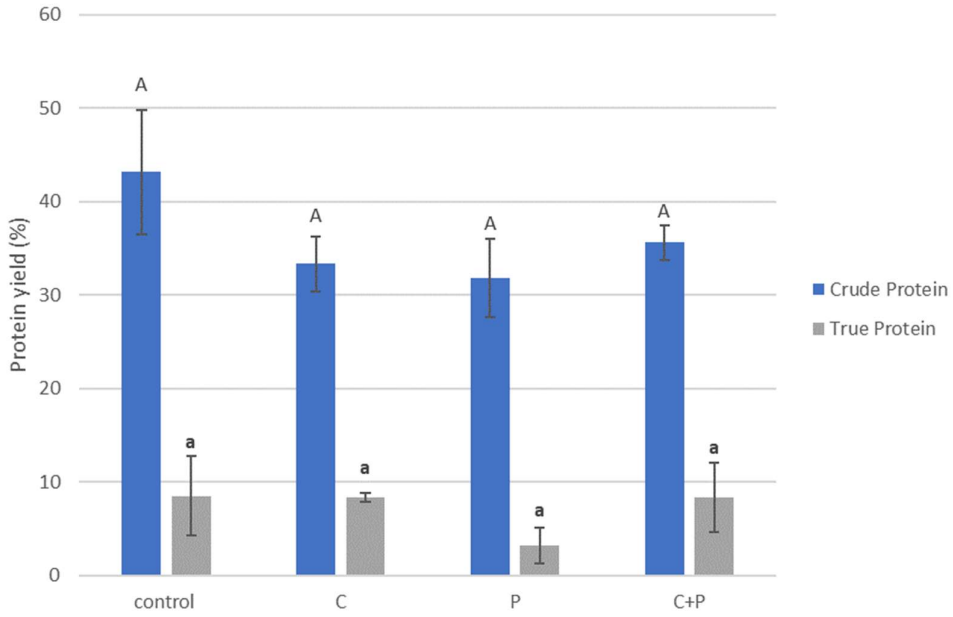

Figure 7: Protein yield after enzyme-assisted extraction. With C = cellulase, P = pectinase, and C+P = cellulase and pectinase. Statistical significance is shown via Compact Letter Display (Capital letters for CP yield, lowercase letters for TP yield). The experiments were done in triplicate.

5.3 High-pressure assisted alkaline extraction

The protein yield from the high-pressure extraction is shown in Figure 8. There were no significant differences in CP or TP yield across different pressures yield across the pressures (CP: p = 0.130; TP: p = 0.686). Although not significant, the crude protein yield does seem to increase with increasing pressure (p = 0.130).

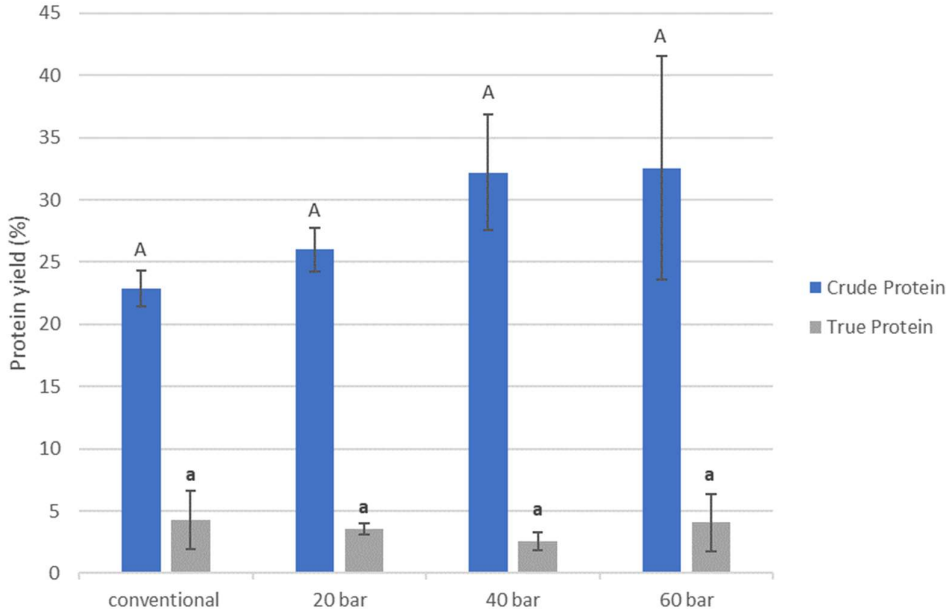

Figure 8: Protein yield after high-pressure assisted extraction. With 20 bar, 40 bar, and 60 bar indicating the target pressure of the treatment. The conventional extraction sample serves as a control. Statistical significance is shown via Compact Letter Display (Capital letters for CP yield, lowercase letters for TP yield). The experiments were done in triplicate.

5.4 Combination of an enzymatic pretreatment and ultrasound-assisted alkaline extraction

The protein yield from the combined enzyme- and ultrasound-assisted extraction is shown in Figure 9. There were no significant differences in CP or TP yield across the different treatments, including the control (CP: p = 0.499; TP: p = 0.448).

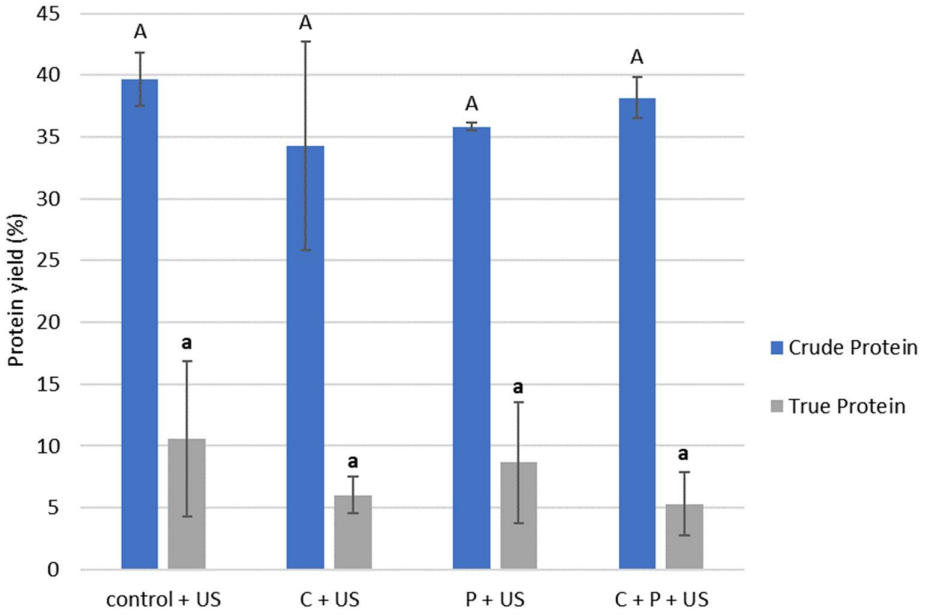

Figure 9: Protein yield after Enzyme and US-assisted extraction. With C = cellulase, P = pectinase, and C+P = cellulase and pectinase. The sonication was performed at an energy level of 10 000 J. Statistical significance is shown via Compact Letter Display (Capital letters for CP yield, lowercase letters for TP yield). The experiments were done in triplicate.

5.5 Comparison between the control samples

The difference in protein yield between the different control samples is shown in Figure 10. The conventional extraction is an alkaline extraction of two hours at pH 9. The control samples were made for the enzyme-assisted extractions; thus, they involved an incubation period at 50°C and pH 5 (0.05 M) for four hours, followed by either the same alkaline extraction (2 h, pH 9) or by an alkaline ultrasound treatment at an energy level of 10 000 J. There is a clear significant difference in the CP yield between the conventional extraction method and the two enzymatic control samples ($p = 0.002$). The CP data from the control samples is roughly double the CP data of the conventional sample. Between the TP yield, no significant differences were observed but the value is roughly doubled when comparing the conventional sample with both the control samples ($p = 0.322$).

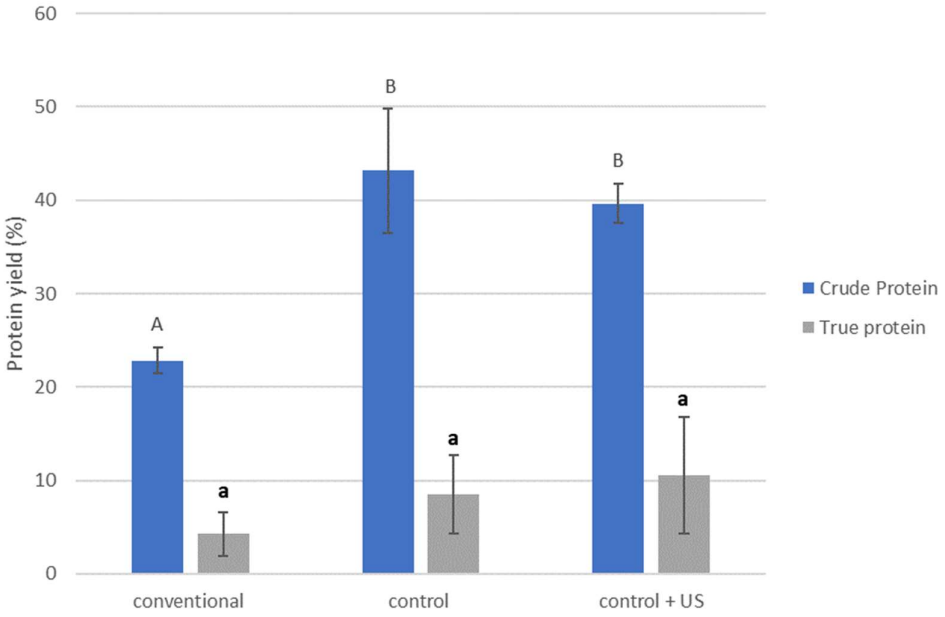

Figure 10: Protein yield compared across control samples. Statistical significance is shown via Compact Letter Display (Capital letters for CP yield, lowercase letters for TP yield). The experiments were done in triplicate.

5.6 Ratio of true protein to crude protein content

The ratio of TP to CP content was compared between all the different groups, and no significant differences were found (P = 0.107). The average ratios of the methods are given in Figure 11. The range of the ratio seems to be roughly 10 to 20%. This means that only 10 to 20% of the extracted nitrogen comes from protein, the rest is non-protein nitrogen.

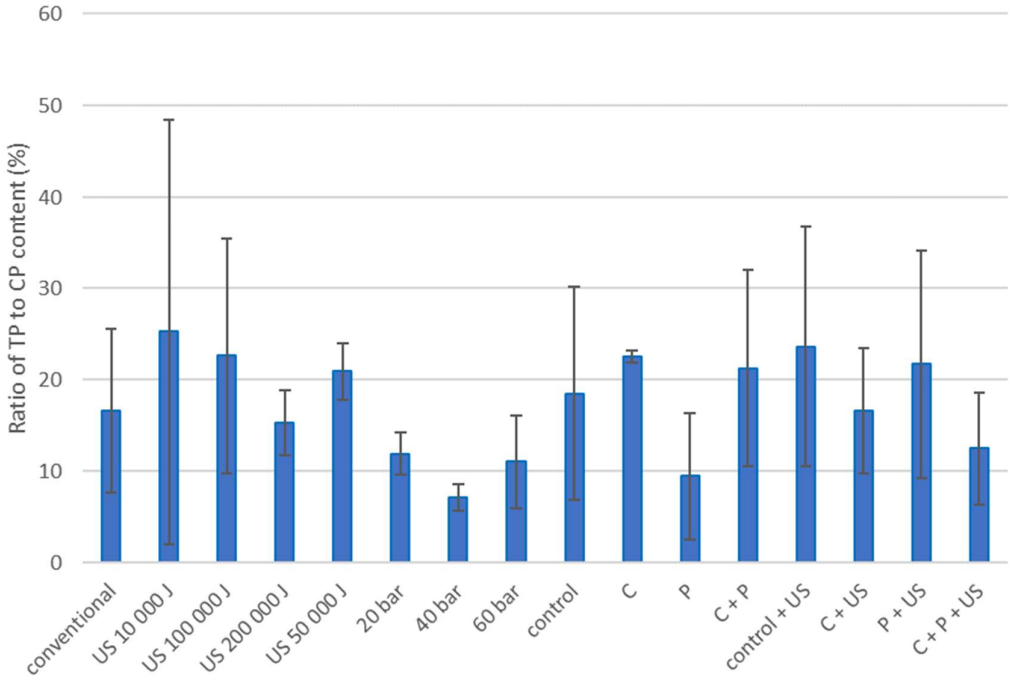

Figure 11: Comparison of the TP to CP content ratio between the different extraction methods. With 10 000 J, 50 000 J, 100 000 J, and 200 000 J indicating the energy level of the sonication. 20, 40, and 60 bar indicating the target pressure of the treatment. And C = cellulase, P = pectinase, and C+P = cellulase and pectinase. When a combination of Enzyme and US treatment was used, the energy level of the sonication was set to 10 000 J. The experiments were done in triplicate.

5.7 Dry matter calculations

The dry matter yield and the CP and TP content of freeze-dried extracts is summarized in Table 4. The dry matter (DM) calculations could only be done with the non-enzymatic samples and the control samples of the enzyme treatments because of a failed batch of freeze-dried samples. Due to this, the DM of the supernatant could not be weighed, and the subsequent calculations could not be executed.

Table 4: Summary of DM yield and protein yield per DM content data across different treatments. DM Yield is expressed as a percentage of the amount of protein that was extracted to the amount of protein in a fresh sample. Protein yield per DM content is expressed as the amount of protein in 100 g freeze-dried supernatant. With 10 000 J, 50 000 J, 100 000 J, and 200 000 J indicating the energy level of the sonication. 20, 40, and 60 bar indicating the target pressure of the treatment. When an Enzyme and US-assisted treatment was executed, an energy level of 10 000 J was set.

	DM yield (%)	Amount of crude protein to the DM weight of the supernatant (g/100g DM extract)	Amount of true protein to the DM weight of the supernatant (g/100g DM extract)
Conventional	5.2 ± 0.3	24.3 ± 1.7	4.0 ± 2.0
US 10 000 J	7.0 ± 0.8	32.3 ± 8.5	7.2 ± 5.0
US 50 000 J	7.9 ± 0.9	24.7 ± 0.5	5.2 ± 0.7
US 100 000 J	9.1 ± 0.9	24.3 ± 2.8	5.7 ± 3.9
US 200 000 J	9.4 ± 0.9	24.3 ± 3.6	3.8 ± 1.4
20 bar	5.4 ± 0.3	26.6 ± 2.9	3.2 ± 0.2
40 bar	5.6 ± 0.3	31.8 ± 3.7	2.3 ± 0.6
60 bar	6.2 ± 1.0	29.5 ± 11.0	3.4 ± 2.2
control	17.1 ± 0.4	13.9 ± 1.9	2.5 ± 0.0
control + US	16.5 ± 1.0	13.4 ± 0.2	4.1 ± 1.0

Statistical analyses were performed to assess the CP and TP content to the DM weight of the supernatant across three different groups: US samples, high-pressure samples, and all non-enzymatic samples. The results revealed no significant differences in the CP content to the DM weight of the supernatant for US samples ($p = 0.181$), high-pressure samples ($p = 0.49$), and all non-enzymatic samples ($p = 0.355$). Similarly, the TP content to the DM weight of the supernatant showed no significant differences across the US samples ($p = 0.665$), high-pressure samples ($p = 0.659$), and all non-enzymatic samples ($p = 0.447$).
However, significant differences between the DM yield were found ($p < 0.001$). The differences in DM yield are shown in Figure 12. When only looking at the conventional and the US samples, there is a roughly linear increase in DM yield starting from the conventional sample to the highest sonication level. Thus, a higher sonication level led to a higher DM yield. No significant differences were observed between the conventional sample and the different high-pressure samples.

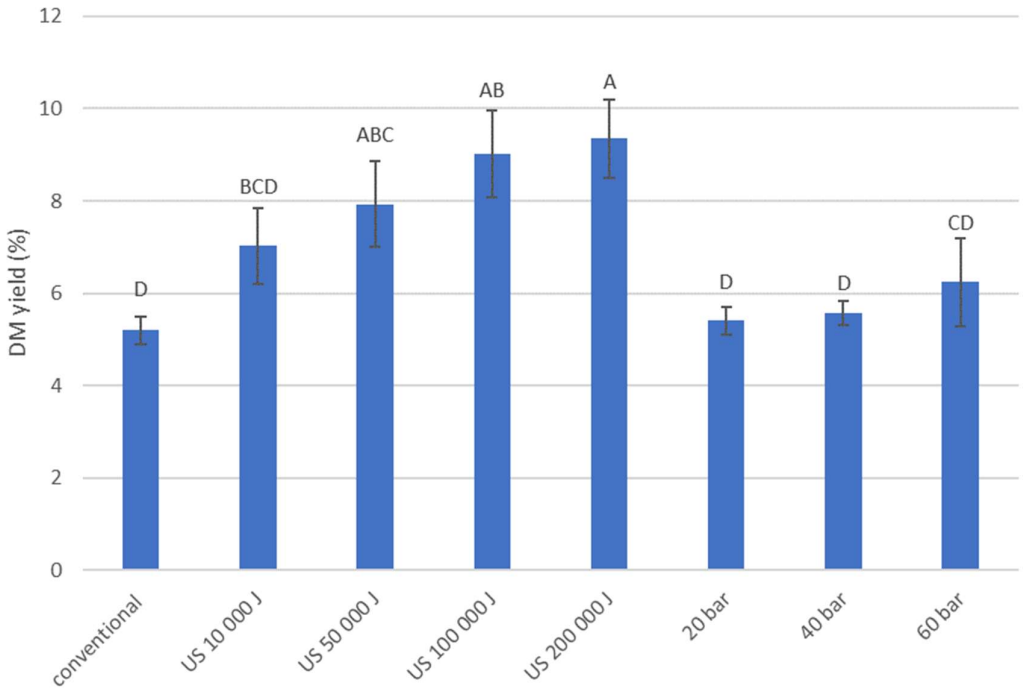

Figure 12: DM yield compared across all non-enzyme extractions. With 10 000 J, 50 000 J, 100 000 J, and 200 000 J indicating the energy level of the sonication. 20, 40, and 60 bar indicating the target pressure of the treatment. Statistical significance shown via Compact Letter Display. The experiments were done in triplicate.

5.8 Microscopic analysis of extraction residues from ultrasound-assisted extractions

Following protein extraction using ultrasound, the pellet after centrifugation was examined using light microscopy. The pictures of different treatments are shown in Figure 13. The matrix disintegrates more and more when using a higher energy level.

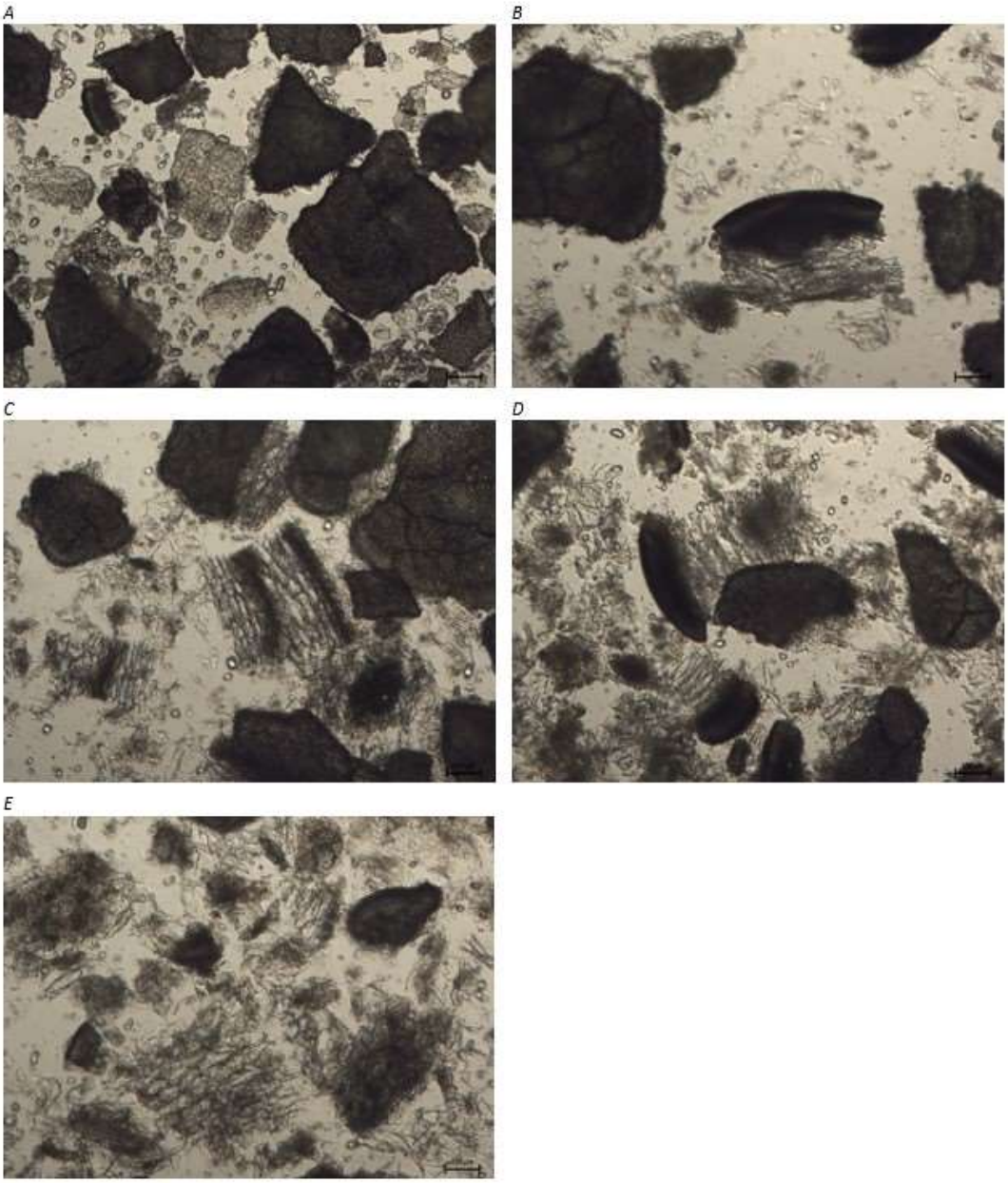

Figure 13: Microscopic pictures of the pellet biomass after the protein solubilization and centrifugation. A, B, C, D, and E are pictures of the pellet after different extraction methods: A. after conventional extraction; B. after US-assisted extraction at 10 000 J; C. after US-assisted extraction at 50 000 J; D. after US-assisted extraction at 100 000 J; and E. after US-assisted extraction at 200 000 J.

6. DISCUSSION

6.1 Non-enzymatic treatments

With conventional extraction, the achieved crude protein (CP) yield was 22.9 ± 1.4 (Figure 5). This is in accordance with the findings of Reinkensmeier et al. (2015), who reported a protein yield of approximately 25% after alkaline extraction at pH 10.68 for 30 minutes from pea testa flour. The liquid-to-solid ratio is also comparable at 2% w/v and the extraction was also performed at room temperature.

The ultrasound (US) treatment had a clear positive effect, approximately doubling both the CP and the true protein (TP) yield of the conventional sample. However, between the different energy levels of the US treatments, no significant differences were found in any of the values, indicating that a longer sonication time does not significantly aid the protein extraction (Figure 5, CP: $p = 0.0152$).

Nevertheless, when looking at the DM yield (Figure 12) of the US samples, there is a clear increase with an increasing sonication time. This indicates that more components dissolve when being subjected to higher ultrasound energy levels. Because the CP yield does not show the same increase, these components are non-nitrogenous. Ultrasound assistance is not only used to aid the protein extraction but also other extractions like fiber or phenol extraction (Vilkhu et al., 2008). Therefore, although the protein yield may not increase, the results indicate that other components, for instance, polysaccharides, were released from the matrix, resulting in a higher dry matter yield.
The TP yield also roughly doubled, similar to the CP yield, but this was not significant ($p=0.478$). The TP yield also showed no difference between the different energy levels. Although the literature suggests degradation of solute structures after sonication with higher applied energy levels (Kumar et al., 2021). This would implicate that after prolonged sonication, the TP yield would decrease due to the degradation of the protein structure.

To get more insight into why the ultrasound treatment was successful, the pellet after protein extraction and centrifugation was observed under a light microscope. There is a clear disruption of the matrix after the use of an ultrasound treatment (Figure 13). There is more and more disruption as the energy levels increase. It looks like the fibers are partly separated due to the treatment. In a study by Morales-Medina et al. (2020) on the impact of microfluidization on the microstructure and functional properties of pea hull fiber, the same defibrillation took place after the microfluidization. Besides defibrillation, a decrease in the particle size of the produced fiber-based suspensions was also observed. Cellulose has a very complex macrostructure with a multi-level organization: cellulose molecule, elementary fibrils, micro- and macrofibrils. The aggregates of macrofibrils are first broken down into individual macrofibrils, and then further into microfibrils. Hence, the particle size reduction can be due to this defibrillation and/or due to a disruption in the remaining cell wall components (Morales-Medina et al., 2020). There is more disruption as the energy level increases, which aligns with the higher DM yield as the energy level increases (Figure

12). The defibrillation causes the release of soluble components stuck between the fibrils. In this case, the components are most likely pectin, hemicellulose, or other components not containing nitrogen, since the protein yield did not increase with an increase in sonication time.

The overnight soaking (SO) preceded by an ultrasound treatment did not affect the protein yield (Figure 6). The crude protein yields were around 35 % (except SO + 10 000 J), similar to the crude protein yields of approximately 40% after only the ultrasound treatment. Since this was a preliminary experiment, it was conducted only once and no statistical analysis was performed. However, the results indicate that soaking the pea hulls overnight at room temperature does not aid the protein extraction.

For the extractions under elevated pressure, no significant differences were observed between the conventional sample and the high-pressure samples (Figure 8, CP: $p = 0.135$; TP: $p = 0.686$). There were also no significant differences between the different pressure levels. The ineffectiveness of this treatment may be due to the relatively low pressure used. Navaf et al. (2023) reported that the usual pressure for high hydrostatic pressure extraction is in the range of 100 to 800 MPa. This is the equivalent of 1 000 to 10 000 bar, far exceeding the highest value of 60 bar used in this study. With 60 bar being the maximum achievable pressure in this laboratory setting.

6.2 Enzymatic pretreatments

Neither the standalone enzymatic treatment nor the combination of enzymatic treatment with ultrasound seems to improve protein extraction (Figures 8 and 10 respectively). This does not have to be the case for all enzymatic treatments, however, as only cellulase and/or pectinase treatments were explored in this study.

6.2.1 Enzyme pretreatments

The lack of an improved yield after the enzyme treatment indicates that the cellulase and/or pectinase enzyme pretreatment were ineffective. The protein yields exhibited even a slight but statistically insignificant decrease after the enzyme pretreatments (Figure 7, CP: $p = 0.063$; TP: $p = 0.135$). Thus, the cellulase, pectinase, and the two enzymes together were ineffective in releasing the protein from the matrix.

In a study by Ansharullah et al. (1997) on rice bran, this ineffectiveness of cellulase to aid protein extraction was also observed. The difference in nitrogen yield between the control sample and the sample with Cellulast®, was 3.26% at 40°C and 0.19% at 50°C. The other parameters were a pH of 3.8, an incubation time of 5h, and an enzyme concentration of 360 NC units. In that same study, when using Viscozyme (120 FBG), the nitrogen yield was over20% more at both temperatures. The enzyme treatments were followed by an extraction at pH 7 for one hour (Ansharullah et al., 1997). In a different study by Perović et al. (2020) on protein recovery from soy grit, the same unproductive result was found as well. The soy grit was treated with different enzymes for one hour, after a pH change, the alkaline extraction went on for one or two hours. No significant difference in yield was found between the control and the samples treated with pectinase,

xylanase, and cellulase separately, followed by an alkaline extraction of two hours. When alkaline extraction of only one hour followed, small, but significant differences were observed between the control and the pectinase and xylanase treatments protein yields. However, significantly higher yields were observed when using a mix of carbohydrases (0.4 U pectinase (from Vinozym), 33 U xylanase (from NS22083) and 20 FPU cellulase (from NS22086)) and when using an Enzyme complex (a commercial cocktail of different enzymes, 0.7 U pectinase, 4 U xylanase, and 20 FPU cellulase) (Perović et al., 2020).

The lack of an increase in protein yield could be due to the possibility that the degradation of cellulose and/or pectin does not result in a higher protein yield. The proteins could be associated with other complex structures like lignin or hemicellulose.
Alternatively, the enzymes could be unable to degrade the pea hull matrix, and therefore be unable to liberate the protein from the matrix. In the production of biofuel from lignocellulosic materials, for example, the enzyme hydrolysis is preceded by a pretreatment to make the cellulose and hemicellulose more accessible to the enzymes. These pretreatments break up the lignin and/or xylan binds in the cell walls, thereby liberating the cellulose and hemicellulose. Possible pretreatments include dilute acid pretreatment or treatment with hot water (Kapsokalyvas et al., 2018).

The product specifications of Celluclast® reveal that the active enzyme in the preparation is β-glucanase (*https://www.sigmaaldrich.com/*, n.d.). This enzyme causes hydrolyzation of β-glucans, these polysaccharides are mostly present in the cell walls of microorganisms or plants like barley and oats (Kaushal et al., 2022). β-glucan is probably also present in pea hulls; prior work has described different parameters that could affect the β-glucan synthesis in pea tissue (Dhugga & Ray, 1991). Hydrolysis by β-glucanase results in smaller β-glucan fragments. These could be an issue since they can interact with the proteins. In a study by Boachie et al. (2021) on pea protein and β-glucans interactions, absorption in the visible light spectrum was used to evaluate biopolymer interactions that cause agglomeration or aggregation. These interactions were tested at different protein to β-glucan ratios. The samples with a ratio of 1:1 and 1:2 protein-β-glucan showed a significant decrease in transmittance after two hours of incubation at room temperature (pH 7), indicating the formation of aggregates or agglomerates between the biopolymers. This same study also explored the ζ-potential of the mixtures. Regardless of the amount of β-glucan (thus also the 1:0.5 ratio), the ζ-potential of the mixtures significantly decreased. The reduced charge leads to decreased repulsion between the biopolymers, resulting in reduced particle stability and increased agglomeration or aggregation (Boachie et al., 2021). This could result in a decreased solubility of the proteins, thus resulting in an indifferent or lower protein yield after using Celluclast©.

ROHAPECT® PTE100 is pure pectin lyase (contact with AB enzymes). This enzyme degrades pectin, forming a shorter pectin molecule and a 4.5 unsaturated galacturonide (Patel et al., 2022). However, the literature suggests that the pectin in the cell walls of pea fiber has a high amount of xylose (Ratnayake & Naguleswaran, 2022). Due to these side chains, there could be a steric hindrance that prevents substrate-enzyme contact.

Although not significant, the crude protein yield decreases by 10% after the different enzyme treatments in comparison to the control (Figure 7, p =0.063).
There may be formations of enzyme-protein complexes that are less soluble due to the non-specific binding of the enzymes. However, since the concentration of the enzyme is relatively low, this probably has a minor impact. Another option is the increased viscosity of the matrix after only partial hydrolysis of the lignocellulosic matrix. The higher viscosity can hinder efficient extraction and separation of the protein.
Alternatively, the intermediate fibrous/polysaccharide debris may interact with the proteins. Proteins and polysaccharides can interact in different ways; possibilities include electrostatic interactions, hydrogen bonding, or hydrophobic interactions (Doublier et al., 2000).
Proteins can form complex coacervates with anionic polysaccharides like pectin or cellulose. This coacervation occurs within an intermediate pH range where the two macromolecules exhibit opposite net charges: specifically, at a pH higher than the isoelectric point (IEP) of the polysaccharide but lower than the IEP of the protein (Gentile, 2020). However, this hypothesis loses some credibility when taking into account the parameters used in the present study. Since the IEP of the proteins is presumably around pH 5 and the pH of the buffer is also 5, this is not in between the IEP of the protein and the IEP of the polysaccharide. A shift in pH to 9 can also alter the charges on the molecules, disrupting these interactions. The other possibility is hydrogen bonding, the -COOH groups of the polysaccharide, along with the $-NH_3$ and -COOH groups on the protein chain, serve as the sources of hydrogen bonding between these two biopolymers (Bandyopadhyay & Ghosh, 2012).
Lastly, the degradation of the cell wall could also release some components that interact with proteins. For example, phenolic compounds can interact with protein, resulting in changes in solubility, thermal stability, and digestibility. These interactions can be covalent or non-covalent and are known to decrease solubility (Ozdal et al., 2013).

6.2.2 Enzyme pretreatments followed by ultrasound

Between the enzyme-treated samples that were followed by a US treatment, there were no significant differences observed between the control and the enzyme treatments and between the different enzymes (Figure 9, CP: p = 0.499; TP: p = 0.448). Additionally, when comparing the enzyme-assisted extraction and the enzyme + US-assisted extraction no significant differences were found between any of the samples, suggesting that the extra US treatment was also ineffective. In comparison to just the enzyme-assisted extraction, however, there may still be a time-saving advantage since similar yields were obtained for two hours of alkaline extraction and about ten minutes of sonication. Although, this is speculative since no alkaline extraction of ten minutes was performed.

6.3 Comparison between control samples

The higher (crude) protein yield after the control samples from the enzyme treatments, in comparison to the conventional alkaline extraction sample, was very surprising (Figure 10). The contrast between the conventional and the control samples for the enzyme treatments appears to be due to the time in the warm water bath as there is no notable difference between the enzyme

control sample and the enzyme control sample that received an additional ultrasound treatment. Only the crude protein values differ significantly, suggesting that only nitrogenous, non-protein components are more easily extracted due to the heat- and mechanical shaking treatment at pH 5. However, the TP values from the control samples are higher than those from the conventional sample, though the difference is not significant ($p = 0.322$).

The first possibility for this increase in protein yield is the inactivation of certain antinutrients within the pea hull powder. Some of them could interfere with the protein solubility. It is known that thermal treatments inactivate protease inhibitors, due to their thermolabile character (Martín-Cabrejas, 2019). In a study by Choi et al. (2019) on 6 different legumes, the protease inhibitor activity was determined after different pretreatments. One of the used treatments was wet heating at 50 or 100°C for 30 or 60 minutes. All the legumes showed a decrease in trypsin inhibitor activity after 60 minutes at 50°C (Choi et al., 2019). Trypsin inhibitors also lose some activity after soaking at room temperature for prolonged periods (Khattab & Arntfield, 2009). Without active inhibitors, the proteases are able to break down proteins into peptides and/or amino acids. These are better soluble in water and thus they could be better extractable (Martín-Cabrejas, 2019).
Besides the inactivation of the protease inhibitors, the conditions in the warm water bath are also quite optimal for some plant proteases. Plant aspartic proteases, for example, are found in seeds and various other plant tissue, are active under acidic conditions, showing an optimum at pH of 4 – 6.5 and a temperature of up to 55°C (David Troncoso et al., 2022). These are very similar conditions to those used during the enzymatic pre-treatment in the present study.
Phytic acid, another antinutrient, acts as a potent chelating agent that aids the formation of complexes with proteins and minerals. Additionally, a significant portion of phytic acid—nearly 25% of its total content in pulses like lentils, broad beans, and peas—is concentrated in the seed hulls (137 – 166 mg/ 100 g DM). A reduction of phytic acid can be achieved by several processing steps, such as soaking, washing, cooking, enzyme treatments and fermentation (Nartea et al., 2023). With soaking being the most popular one, in acidic conditions (pH 2.5 – 4.5), soaking could help to further decrease the phytic acid concentration because, at these conditions, the endogenous phytase enzyme is activated. This enzyme catalyzes the hydrolysis of phytic acid, the hydrolyzed forms have a smaller affinity to bind the metal ions and form complexes (Sarkhel & Roy, 2022). The non-significant rise in TP yield after the warm water bath could be because only non-protein nitrogen components are liberated from the matrix. Alternatively, the proteases may have degraded the released proteins, preventing them from precipitating with TCA.

Besides the possible impact of anti-nutritional factors, prolonged soaking in warm, acid water could also affect the lignocellulosic matrix. Dilute-acid pretreatment is a treatment often used in the production of biofuel. The treatment is done to enhance the bioavailability of the sugars to the micro-organisms, thus enhancing the ethanol yields. The cellulose becomes more accessible by undergoing decrystallization, microfiber swelling, depolymerization, and ultimately conversion into monosaccharides. Because of the untightened structure of the cellulose, it could be easier for the proteins to dissolve. An important caveat to this theory is that most pH and temperature parameters for this pretreatment in literature are harsher than 50°C and a pH of 5, the treatment

time is, however, shorter and the substrate loading is higher (du Pasquier et al., 2023; Noureddini & Byun, 2010). However, it could be argued that for protein extraction, no complete hydrolysis is necessary. A looser structure of the matrix could also already aid in the extraction of protein.
In a study by Noureddini & Byun (2010) on corn and distillers' grains, the effect of the different parameters was researched. Pretreatment reactions varied by temperature (120, 140°C), biomass loading (5, 10, 15, 20% w/v), sulfuric acid concentration (0.5, 1.0, 1.5 vol.% using 98% acid), and between 0 and 60 minutes. The hydrolysis was evaluated by the number of monomeric sugars formed. The highest yields were observed at lower substrate loadings, higher acid concentrations, and at a temperature of 140°C. An upward trend of the yield, as the treatment time increases, was also reported (Noureddini & Byun, 2010).

The pH of the control samples is set at 5 using a sodium acetate buffer with a concentration of 0.05M, resulting in a higher ionic strength due to the presence of Na^+, H^+, and CH_3COO^- ions. Ionic strength can impact protein-protein and protein-water interactions, and subsequently protein solubilization (Li & Xiong, 2021). In a study by Sarigiannidou et al. (2022) on pea protein isolate, the hydrodynamic diameter (D*h*) of soluble pea protein hydrolysate (hydrolyzed pea protein isolate) was measured at different NaCl concentrations (pH 7.2). The D*h* decreased significantly at lower concentrations (0.1 and 0.2M NaCl) compared to a salt concentration of 0M. This decrease is attributed to the salting-in effect, which enhances solubility. Conversely, at a higher ion concentration (0.4M NaCl), the D*h* increased, leading to a decrease in solubility (Sarigiannidou et al., 2022). The mechanism behind the salting-in effect is highly speculated in literature. The phenomenon is predominantly present near the iso-electric point (IEP) and the effect might be attributed to an increased solvent affinity for the protein surface. Near the IEP, the diminished charge on the protein impacts the amount of water associated with it. Ions may bind to the proteins through ion-dipole interactions, thereby elevating their net charge and, consequently, their solubility (Maurer et al., 2011).

Lastly, the mildly acidic pH and the warm temperature could also result in slight conformational changes in the protein structures or may result in the disruption of certain protein-protein interactions. These structural changes could result in different interactions with the solvent—in this case, water. Denatured proteins also have a higher surface area, thus being more vulnerable to enzymatic hydrolysis as discussed above. Pre-existing research suggests that the temperature at which pea protein undergo denaturation is between 75 and 85°C (pH 7) (Kornet et al., 2022). Kornet et al. (2022) investigated the denaturation temperatures of different pea protein fractions. The albumin-rich fraction had a peak denaturation temperature of 87.7°C and a denaturation onset temperature of 81.8°C. these values for the globulin-rich fractions are respectively 82.3°C and 72.5°C. In addition to that, if the temperature is increased slowly, protein conformations generally remain the same until an abrupt loss of structure (Nelson & Cox, 2005). Thus, making this theory of 'slight' denaturation disputable. However, the compatibility between pea hull protein and pea protein is unknown, thus, the denaturation temperatures could be different.

6.4 Ratio of true protein to crude protein content

Another surprising result is the difference between the crude protein and true protein data. Only around 10 to 20% of the crude protein content (Figure 11), is determined as true protein. It is also worth noting that this ratio is consistent across treatments, no significant differences were observed ($p = 0.107$).

The stark difference between the CP and TP yield could be explained by the data used for the TP yield calculations, particularly the TP content of the raw material, which was utilized in Formula 6. This data was obtained by performing the same TCA precipitation process outlined in the materials and methods section. Pea hull powder was mixed with 20% TCA solution, the samples were placed in an ice bath and subsequently centrifuged. The resulting pellet was then analyzed using the Kjeldahl method to determine the true protein content of the material. This value was subsequently used to calculate the TP yield. Consequently, only nitrogen compounds that dissolved in the TCA solution were removed, leaving the nitrogen components embedded within the raw material's structure in the pellet, thereby being accounted as TP. This might result in an overestimation of the TP content of the raw material, leading to an underestimation of the TP yield of the samples.
Non-protein nitrogen (NPN) components in the tissue could include alkaloids, chlorophyll, certain glycosides, amides, free amino acids, nitrogenous fats, nitrates, B-vitamins, purines, pyrimidines, and ammonium salts (Ezeagu et al., 2002). The amino acids, for example, are transported exclusively through the phloem from the endosperm, once they are in the seed coat, they undergo elaborate metabolic conversion and are released into the apoplast, the space outside of the membrane in the cell wall (Moïse et al., 2005).

The pea hulls could be rich in nitrogenous components other than protein. The literature on legumes reports that there is quite a high percentage of NPN in legumes. This NPN is especially present in the seed coat, with a ratio of 22 to 28% of the total nitrogen in the hulls of gram greens having a non-protein origin (Sashikala et al., 2015). This nitrogen can come from components like peptides and free amino acids that can be water-soluble. The NPN can be estimated by subtracting the TP content from the CP content. In a study on NPN by Periago et al. (1996), the total nitrogen content of pea powder was estimated to be 4420 ± 80 mg/100g DM, the protein nitrogen as 1700 ± 35 mg/100g DM, and thus the NPN as 2720 ± 10 mg/100g DM. Therefore, the ratio of TP to CP in pea powder would be around 40%. The protein nitrogen value was obtained by alkaline solubilization, with different rounds, and TCA precipitation (Periago et al., 1996). The 40% figure substantially exceeds the 10 to 20% TP to CP ratio of the extract. However, when this figure is evaluated in the context of the raw material composition - documented as 89.7% in section 4.3.2 - it appears significantly lower. This discrepancy lends support to the first theory that there may have been an erroneous determination of the TP content of the pea hull powder.

For a more accurate determination of TP in the raw material, an alternative method to TCA precipitation should be used. It is generally agreed that amino acid quantification is the most accurate method. The amino acids are detected after hydrolysis via high-pressure liquid chromatography. Hydrolysis conditions must balance between incomplete hydrolysis and amino

acid degradation. However, with this procedure, the free amino acids are also accounted for as true protein (Sägesser et al., 2023).

The low TP content could also be explained by a lack of precipitation of small molecular weight peptide materials by TCA (Xiong et al., 2016). Since the nature of the proteins present in the pea hull is not known, they could also be of a smaller molecular weight and thus harder to precipitate.

Generally, the observed TP values are always rather low and often have a high standard deviation. Consequently, there was never a significant difference observed between any of these values. Due to this, the true protein yield was not focused on in this discussion. The high standard deviations could be due to the very low amounts extracted and/or precipitated using TCA, due to the hypotheses outlined above.

7. CONCLUSIONS AND FUTURE PERSPECTIVES

This research aimed to explore more efficient ways for protein extraction from pea hulls. The biggest obstacle to this extraction is the very rigid cell wall structure. Structural proteins, or other proteins embedded in cell walls, are therefore hard to extract, as are proteins in the cell itself. This barrier can be surmounted by finding ways to disrupt the cellular structure. The literature describes a wide range of extraction treatments and technologies to aid this disruption. In this study, ultrasound, enzymes, a combination of ultrasound and enzymes, and high-pressure treatments were explored.

The ultrasound treatment aided the crude protein extraction significantly. All the different energy levels roughly doubled the protein yield in comparison to the conventional alkaline extraction. However, higher energy levels of sonication do not result in better protein extraction yields. This is a positive conclusion regarding the industry since higher energy levels require more energy. Consequently, being more costly because of both a higher energy consumption and an increase in process time. Because of the higher energy consumption, longer sonication times also increase the environmental burden.

The high-pressure assisted protein extraction did not result in a higher protein yield in comparison to the conventional extraction. Since the pressures used in the literature on high-pressure assisted protein extractions are much higher (> 1000 bar), it might be interesting to explore protein these ultra-high-pressures.

As for the enzymatic-assisted extraction (cellulase and/or pectinase), the results were also less promising. The enzyme-treated extractions did not result in a higher protein yield in comparison to the control. Thus, the used enzymes did not aid the protein extraction. For future studies, it could be interesting to explore the effect of different enzymes than the commercial cellulase and pectinase used here. Since the cell wall of the pea hull cells contains some xylose-rich pectin, it could be useful to try β-xylosidase. This enzyme can hydrolyze the bonds between the galacturonic acid of the pectin and the xylose, but also the bonds between the xylose molecules (Martínez et al., 2004). Other enzymes that first cleave the different side chains that can be present in pectin could also be explored. When the side chains are removed, the pectinases may be more effective because of the lack of steric hindrance, thus increasing the substrate availability for the enzyme.
Exploring different types of cellulase preparations could also be worthwhile since the enzyme β-glucanase releases β-glucan fragments, which could interfere with the protein solubility.
The enzyme-assisted extraction followed by an ultrasound treatment did not improve the protein yield compared to the enzyme-assisted extraction followed by alkaline extraction. This suggests that the US treatment has the same effect as performing an extraction of two hours on a shaker. This could be considered positive since the sonication time of the ultrasound treatment was only around ten minutes, which is considerably shorter than the two hours of shaking extraction. As a trade-off, the US treatment does use more energy and will probably be more costly to operate. It should be noted that a shaking alkaline extraction of ten minutes after the enzyme incubation was not tested, so this time-saving advantage of the ultrasound treatment is speculative.

Furthermore, it may be useful to determine whether there is any degradation of the cell wall due to enzyme activity. Measuring the concentrations of specific monosaccharides, such as glucose, in the extraction solution could indicate the extent of hydrolysis. With this information, theories on why enzyme-assisted extraction did not work could be either backed or debunked.

To increase the yield after enzyme extraction, the availability of the substrate to the enzyme is of great importance. As previously discussed, a higher availability could be achieved due to the removal of certain side chains or softening of the matrix. An ultrasound pretreatment before the enzyme treatment may also be an option. In this case, the US treatment might disrupt the cellular structure first. This would increase the surface area, consequently revealing more substrate to the enzymes. Sequential use of enzymes could also improve the protein yield. Since cellulose and hemicellulose are embedded in a pectin matrix, it may be useful to degrade pectin first, before adding cellulose and hemicellulose degrading enzymes.

Perhaps one of the most notable outcomes of this study is the substantial rise in crude protein yield observed after immersing the pea hull powder in water for four hours at a pH of 5 and 50°C, which was then followed by either a two-hour alkaline extraction or a 10-minute US treatment, compared to merely undergoing a two-hour alkaline extraction alone. This could be more deeply explored in the future. The implications of this could be quite significant, especially considering that one of the challenges with enzymatic treatments is the high cost of enzymes. Since the enzymes did not improve protein extraction, this cost could be avoided.
It would be interesting to determine the protein yield after a longer incubation time, or a shorter incubation time (more cost-effective). The impact of different soaking temperatures or acidity levels could also be explored to better understand what happens in the matrix. Exploring various ions and their concentrations could offer insights into the hypothesized salting-in effect due to ionic strength.
One of the theories behind the improved yield after soaking is the softening of the matrix. After the warm soaking, an enzyme treatment could be explored again, it may be more successful because the components are more easily accessible, thus facilitating enzyme-substrate complexes.

True protein values in this study are quite unreliable because of their often high standard deviations. Taking larger samples could be a solution but ultimately it would be more useful to do a characterization of the proteins present in the pea hull. As previously discussed (section 6.4), their molecular size can play an important role in their ability to precipitate with TCA. Such characterization could be done by electrophoretic techniques like SDS-PAGE. Since SDS-PAGE can be semiquantitative too, it could be used for a more accurate estimation of the true protein amount in a sample. If a quantitative method is desired, HPLC could be an option too.

To complete a full protein isolation, the solubilization step needs to be followed by a precipitation step. The precipitation conditions need to be studied and optimized. The properties of the proteins isolated in this way should also be investigated. Proteins are often used in the food industry for which their functionality is extremely important. Different functional properties can be desired in

different food systems, examples include water binding capacity, as well as foaming and emulsification abilities.

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9. APPENDICES

Appendix one

Protein yield is the ratio of protein that was extracted to the amount of protein in a fresh sample. Content being expressed as g protein to 100 g supernatant. With 10 000 J, 50 000 J, 100 000 J, and 200 000 J indicating the energy level of the sonication. With C = cellulase, P = pectinase, and C+P = cellulase and pectinase and with 20, 40, and 60 bar indicating the target pressure of the treatment. When an Enzyme and US-assisted pretreatment was done, an energy level of 10 000J was set.

	Crude protein yield (%)	True protein yield (%)	Crude protein content (x 10^{-2} g/100g)	True protein content (x 10^{-2} g/100g)
conventional	22.86 ± 1.43	4.27 ± 2.35	3.756 ± 0.233	0.626 ± 0.335
US 10 000 J	41.01 ± 10.84	9.78 ± 6.15	6.089 ± 1.708	1.283 ± 0.773
US 50 000 J	35.45 ± 3.60	8.29 ± 1.64	5.384 ± 0.334	1.133 ± 0.241
US 100 000 J	39.63 ± 4.94	10.20 ± 6.35	6.011 ± 0.769	1.407 ± 0.932
US 200 000 J	40.77 ± 3.14	7.05 ± 2.11	6.575 ± 0.212	1.012 ± 0.256
20 bar	26.00 ± 1.77	3.55 ± 0.48	2.752 ± 0.348	0.345 ± 0.033
40 bar	32.19 ± 4.65	2.59 ± 0.72	3.492 ± 0.667	0.254 ± 0.081
60 bar	32.56 ± 8.97	4.05 ± 2.30	3.664 ± 1.417	0.424 ± 0.278
control	43.16 ± 6.63	8.48 ± 4.22	6.775 ± 1.066	1.192 ± 0.590
C	33.35 ± 2.95	8.37 ± 0.50	5.041 ± 0.301	1.135 ± 0.035
P	31.81 ± 4.17	3.14 ± 1.92	5.361 ± 0.591	0.482 ± 0.301
C+P	35.61 ± 1.86	8.31 ± 3.76	5.762 ± 0.338	1.202 ± 0.540
control + US	39.65 ± 2.14	10.57 ± 6.25	6.432 ± 0.291	1.545 ± 0.907
C + US	34.30 ± 8.46	6.03 ± 1.45	5.227 ± 1.279	0.826 ± 0.210
P + US	35.83 ± 0.34	8.65 ± 4.92	6.019 ± 0.458	1.332 ± 0.852
C + P + US	38.16 ± 1.65	5.31 ± 2.56	5.805 ± 0.346	0.714 ± 0.309