VILNIUS UNIVERSITY
LIFE SCIENCES CENTER

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Molecular Biotechnology study programme

Master Thesis

DEVELOPMENT OF BIOCATALYSIS BASED TECHNOLOGY FOR THE HYDROLYSIS OF PLANT-DERIVED PROTEINS

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ABBREVIATIONS

AA/BAA – acrylamide/ bis-acrylamide

AAs – amino acids

Amp – ampicillin

APS – ammonium persulphate

ATR-FTIR spectroscopy - attenuated total reflectance Fourier transform infrared spectroscopy

BMGY – buffered glycerol-complex medium

BMMY – buffered methanol-complex medium

BSA – bovine serum albumin

CFU – colony-forming unit

DH – degree of hydrolysis

DMC – N,N-dimethylcasein

DTT – dithiothreitol

EDTA – ethylenediaminetetraacetic acid

G418 – geneticin

GGA – Golden Gate assembly

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

LB medium – Luria-Bertani medium

Ori – origin of replication

PMSF – phenylmethylsulfonyl fluoride

POE-PCR – prolonged overlap extension polymerase chain reaction

PVDF – polyvinylidene fluoride

SDS-PAGE – sodium dodecyl-sulfate polyacrylamide gel electrophoresis

SRP – serratopeptidase

TAE – tris-acetate-EDTA buffer

TEMED – N,N,N′,N′ -Tetramethylethylenediamine

Tm – primer melting temperature

TNBS – 2,4,6-trinitrobenzene sulfonic acid

Tris – tris(hydroxymethyl)aminomethane

YEPD – yeast extract, peptone, dextrose containing medium

YNB – yeast nitrogen base

INTRODUCTION

The global population is rapidly increasing and is forecast to reach around 9.7 billion by 2050. Huge efforts are being made to boost the search for affordable, sustainable, and high-nutrition food. Proteins are the major nutritional component of daily diet, as they play a key role in various biological processes in humans. High quality proteins can be obtained from animal sources; however, the production of animal-origin proteins is a major contributor to climate change, pollution, deforestation and biodiversity loss. The concerns related to animal protein production have prompted the exploration of alternative protein sources. Plant-based proteins may provide a more environmentally friendly, affordable, and sustainable substitute for animal-based proteins. It is projected that by the end of 2027 the plant-based protein market will reach 14.4 billion USD. Plants provide high levels of proteins, a range of vitamins, minerals and antioxidants. In addition, plant-based protein production requires less energy and water, compared to animal-origin proteins. However, to enhance plant-derived proteins functionality to their full potential novel processing methods are required. Protein hydrolysate is the most nutritious form of plant-derived proteins. It contains a mixture of short-chain peptides and free amino acids, which are more readily absorbed by the body. Short chain peptides, formed during hydrolysis, may exhibit various bioactivities. It may act as a high blood pressure controller, anticoagulant, antioxidant, and has a positive effect on reducing the risk of cardiovascular disease. Various methods can be applied for the production of protein hydrolysates, such as chemical hydrolysis, enzymatic hydrolysis or fermentation. Enzymatic hydrolysis is preferred, because it avoids chemical or microbial contaminations of the proteins, and is performed under mild reaction conditions, specificity of the enzymes ensures the precise process control, and it is an environmentally safe process.

This study focuses on the investigation of the enzymatic hydrolysis process using alfalfa meal as a substrate, with its protein content of approximately 15% in stems and 35% in leaves. The alfalfa plant emerges as a promising source for the development of plant-protein hydrolysates.

Aim of the study: To develop a technology for plant-based protein hydrolysis via biocatalysis.

Objectives:

- To develop ATR-FTIR spectroscopy-based method for the assessment of hydrolysis process.
- To establish enzymatic hydrolysis parameters of alfafa meal by commercial protease.
- To develop an expression system for heterologous recombinant protease in *Pichia pastoris* yeast.

1. LITERATURE REVIEW

1.1 Bioeconomy – what is it?

There is estimated that the global population will reach 9.7 billion people in 2050[1]. To be able to accommodate a growing global population, as well as rapid depletion of many resources, mounting environmental issues, and climate change, Europe must switch to an entirely different approach for producing, consuming, storing, recycling, and discarding biological resources. Solving the mentioned issues, the bioeconomy is foreseen to be a key player. Bioeconomy is the part of the economy that produces renewable raw materials and processes these raw materials and biological waste into value-added products.

The EU Bioeconomy Strategy is a plan proposed by the European Commission to ensure a sustainable and competitive bioeconomy for Europe. The strategy focuses on transitioning to a circular economy, where resources are continuously reused, reducing the amount of waste and greenhouse gas emissions, and increasing the use of bio-based materials and renewable energy sources. It also emphasizes the importance of investing in research and innovation, to promote the development of new technologies and solutions that can help to reduce resource use and ensure biodiversity and environmental protection. The strategy constitutes of the five main objectives (**Fig.1.1**).

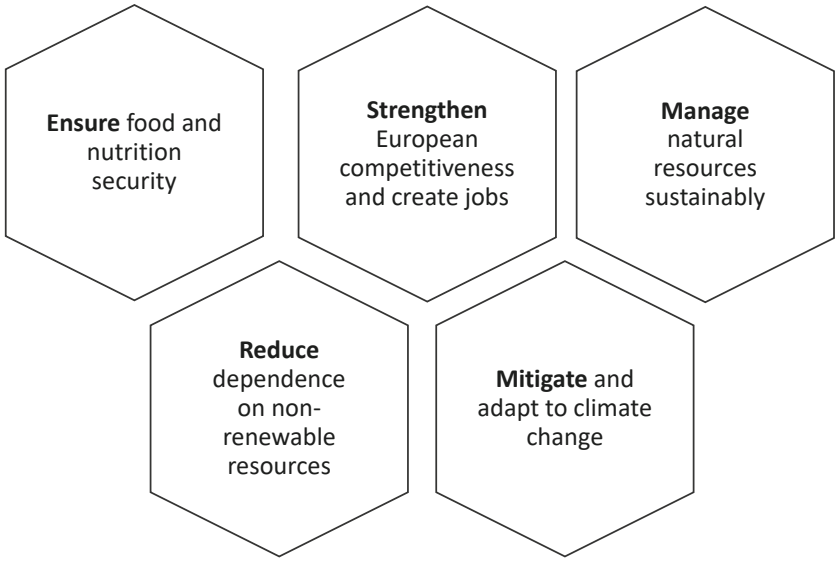


Fig.1.1 The main objectives of the EU Bioeconomy Strategy

Food and feed systems embrace the largest niche of bioeconomy (FAO, 2022). It is projected that food and feed production needs to increase by 70% by 2050 to meet the world's food demand. At this point, big efforts are being made to boost the search for sustainable and high-nutritional food, especially a lot of attention is given for exploring an alternative protein sources. Sources of alternative

[1] Food and agriculture organization of the United Nations https://www.fao.org/in-action/sustainable-and-circular-bioeconomy/resources/news/details/en/c/1507553/

proteins varies from microbial proteins (microalgae and mycoproteins), insect-based proteins, and cell-based/cultivated meat to plant-based proteins (meat substitutes, dairy alternatives) and fungi. The interest in this niche sector has been rapidly growing in recent years due to several factors, including changing consumer tastes, increased environmental and ethical concerns around traditional animal-origin protein production. These factors go toward replacing traditional animal proteins by alternative ones. Plants are one of the most potential sources for alternative protein production.

1.2 Animal-based and plant-based proteins in food

Proteins are one of the most important nutrients that are required for critical functions in the cells, tissues, organs, and all systems. Insufficient amount of proteins in human diet, can lead to wide-ranging side effects, such as stunting, anemia, physical weakness, edema, vascular dysfunction, and impaired immunity (Ives & Brickley, 2010). Proteins provide nitrogen, hydrocarbon skeletons, and sulfur, which are essential components of organisms, and cannot be replaced by any other nutrients, including carbohydrate and lipids, because neither nitrogen nor sulfur are made in the body (Wu et al., 2016). To maintain good health and well-being, it is crucial to get an adequate amount of proteins in the daily human diet.

Proteins could be obtained by animal or plant origin. Proteins consist of amino acids (AAs) and the composition of them depends on protein origin. AAs composition determines both – the structure and function of proteins (Berg., 2002). In nutrition value, amino acids are grouped into the essential AAs and non-essential or dispensable AAs (Fig.1.2). Essential AAs cannot be synthesized in human body and must be obtained from food sources; non-essential AAs are obtained through dietary sources and are synthesized in the cells of the human body.

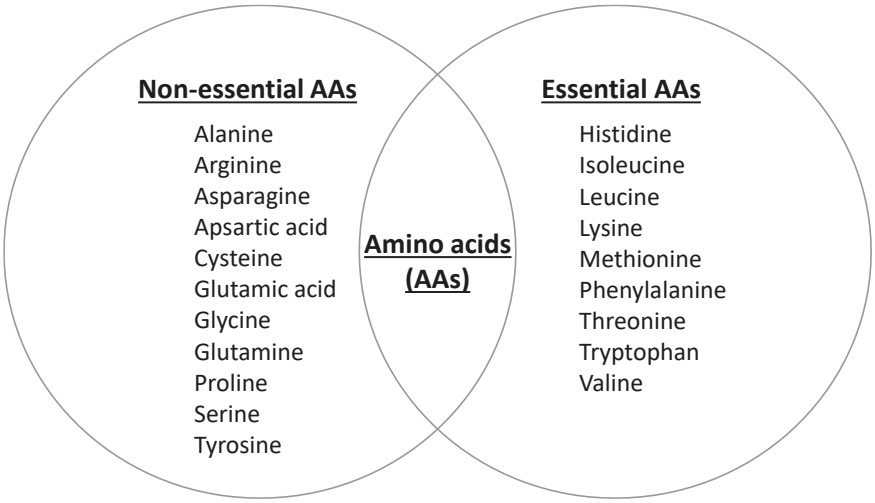


Fig.1.2 Non-essential and essential amino acids

After consumption, food proteins are broken down by digestive enzymes into an absorbable form – a single AA or peptides consisting of several AAs. Digestibility, the quality factor of proteins, is considered as the proportion of ingested AAs that can be made available for utilization by the body

after digestion and absorption. Digestibility depends on the protein structure, thermal processing intensity, and presence of some compounds that are prejudicial to protein digestion, the so-called antinutritional factors (Sá et al., 2020). *The Protein Digestibility Corrected Amino Acid Score* (PDCAAS) and the *Digestible Essential Amino Acid Score* (DIAAS) are two main indices used to measure the ability of proteins to meet the body's amino acid requirements (FAO, 2014). The values of these indicators vary depending on the protein source.

Comparison of traditional animal and alternative plant-based protein sources

Traditional animal-origin proteins are one of the main dietary protein sources. It contains all of the nine essential amino acids and are considered to have "complete protein packages" that could provide daily rate of proteins (Shams-White et al., 2018). Even though animal-origin proteins are good sources for food production, they have a negative environmental impact. For example, the production of 1 kg of high-quality animal-origin proteins requires to feed 6 kg of plant proteins to livestock, which makes an impact on land and water resources, as well as increased amounts of greenhouse gas emissions (Henchion et al., 2017). The use of cows, pigs, and other animals for food, as well as livestock feed, is responsible for 57% of all food production emissions, while only 29% coming from the cultivation of plant-based food (Xu et al., 2021). In addition, long-term animal meat consumption can increase the risk of chronic diseases. It is associated with the high level of saturated fatty acids found in animal-origin products, that could provoke certain lifestyle illnesses such as cardiovascular disease, obesity, type II diabetes mellitus, increased levels of low-density lipoprotein (LDL) cholesterol or cancer of bowel (Nadathur et al., 2017; Richter et al., 2015). The healthy and environmental concerns about the animal-origin proteins boosted the search of alternative protein sources.

The popularity of plant-based proteins has grown in recent years, largely due to the health benefits they offer over animal foods. Plant-based protein consumption is associated with reductions in risk for developing chronic diseases (García et al., 2013). Plant origin proteins are generally lower in calories, fat, and cholesterol. It can provide a range of vitamins, minerals, and antioxidants. Also, it is rich source of fiber, carbohydrates, oligosaccharides, and polyunsaturated fatty acids, so, it plays an important and valuable role in human nutrition. Plant-based proteins are more environmentally friendly than animal-based proteins, as they require less energy and water to produce. Moreover, plant-based proteins have a low risk of food-borne disease, as animal proteins could more often contain bacteria and parasites. For these reasons, plant-based proteins are an excellent alternative to animal-based proteins for those looking for a healthy and sustainable diet.

Plant-based protein market

The valuable advantages of plant-origin proteins and changes in consumer's dietary lifestyles have resulted in the increasing market of plant-based protein production. According to "Markets and Markets", the global plant-based protein market is expected to reach 17.4 billion USD by the end of 2027, while currently it is estimated to be valued at USD 12.2 billion in 2022[2]. According to the source of plant-based proteins, the soy origin products are estimated to account for the largest market share during the forecasted period. Soy is one of the few sources of plant-based proteins, which could provide a complete nutritional protein package that contains all essential amino acids.

Based on the protein processing and extraction techniques, several types of protein products are distinguished: concentrate, isolate and hydrolysate.

Protein concentrate is usually obtained by the removal of the non-protein components, mainly carbohydrates, it contains lower content of proteins than isolates, but it often retains nutrients found in the original protein sources, such as anti-inflammatory molecules, antioxidants, vitamins, and minerals. *Protein isolate* is a purified form of protein powder with minimal content of other macronutrients (Yalcin & Celik, 2007). The last form is *protein hydrolysates* produced from protein isolates or protein concentrate *via* enzymatic, chemical hydrolysis or fermentation. After treatment hydrolysates become a mixture of short chain peptides and amino acids (Gençdağ et al., 2021). Comparing market share based on the forms of plant-based proteins, currently the segment of concentrates is accounted for the largest market share[3]. Additionally, protein hydrolysates are in the high demand for their potential use in functional food due to their bioactive peptides, improved solubility, and resistance to oxidative agents such as metal ions (Sari et al., 2015).

Even one of the biggest global biotechnology companies "Novozyme", which focus is the research, development and production of industrial enzymes, microorganisms, and biopharmaceutical ingredients, is also increasing its investment into the alternative protein production. In 2021, the company invested 300 million USD in new plant-based production line. Specifically, the major attention is given to enzyme solutions that can be used for the production of alternative protein food and beverage products[4]. Currently, the company is orienting its focus on unique ingredients which could enable better plant-based food and beverage products, and proteins for specialized application in nutrition and health.

[2] https://www.marketsandmarkets.com/Market-Reports/plant-based-protein-market-14715651.html
[3] https://www.researchandmarkets.com/reports/5264248/global-plant-based-protein-market-by-source-soy?utm_source=GNOM&utm_medium=PressRelease&utm_code=fx35fm&utm_campaign=1733926+-+Global+Plant-based+Protein+Market+to+Reach+%2417.4+Billion+by+2027&utm_exec=como322prd
[4] https://www.foodnavigator-usa.com/Article/2022/09/19/novozymes-ramps-up-investment-in-alternative-proteins-our-journey-has-just-begun?utm_source=copyright&utm_medium=OnSite&utm_campaign=copyright

1.3 Benefits of plant-based protein hydrolysates

Bioactivity of protein hydrolysates

Many plants have a lot of beneficial qualities, which are not far-fetched from their protein components. Protein or peptide which provides a health benefit beyond its basic nutritional functions, may be termed as a functional ingredient and can be used in functional food (García et al., 2013). Bioactive peptides (BP) are obtained by the hydrolysis process, generated via fermentation, enzymatic or chemical hydrolysis of their parent proteins (Ashaolu et al., 2020). Depending on the hydrolysis process conditions and amino acids composition of plant protein, it could be converted into various short chain peptides with different bioactivity (Fig. 1.3).

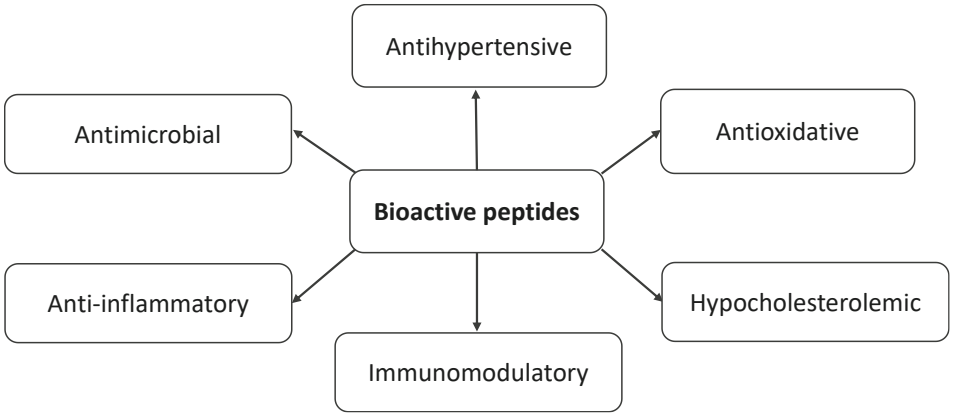


Fig. 1.3 Bioactivity of plant-based peptides. Adapted from Marciniak et al., (2018)

Antihypertensivity is the main bioactivity found in plant-derived peptides. There are different biological pathways that regulate blood pressure in living organisms, but peptides mainly act on rennin-angiotensin system. Angiotensin converting enzyme (ACE) helps to regulate blood pressure by converting hormone angiotensin I to angiotensin II, which raises the blood pressure (Okamoto et al., 1995). Some commercial ACE inhibitors are powerful drugs, but they can cause various side effects like coughing, skin rashes or problems with taste. Plant-derived peptides that act as an ACE inhibitors might be a better alternative to synthetic drugs because they do not cause any serious side effects (García et al., 2013). The most active peptides were found in plant-origin sources such as legumes (chickpea and soybean), wakame seaweed, cereals (maize and wheat), and sesame (Du et al., 2013; Malaguti et al., 2014; Okamoto et al., 1995).

Another important BP group found in plant-based protein hydrolysates is antioxidant peptides. Oxidative stress occurs when there is an imbalance between oxidizing agents and natural antioxidants which could detoxify them. It has been linked with aging, cell death, tissue damage and serious diseases such as cancer (Malaguti et al., 2014). The presence of antioxidant compounds in plant-based proteins is of great interest not just for health benefits, but also due to their relation with organoleptic food properties. Radical-mediated lipid oxidation in foods can produce rancid flavors, food discoloration, and toxic undesirable compounds, which can lead to quality deterioration and shortened

shelf life (Chen et al., 2019). Adding synthetic antioxidants is a common practice in food processing, but it has disadvantages of high costs and the potential toxicity risk. Consequently, many researchers are searching for natural antioxidants and plant-based peptides are one of the potent sources that may protect the body and foodstuffs from free radicals and retard their deterioration. The most studied plant sources of antioxidant peptides are black soybean, corn, moringa oleifera seed, oat bran, rapeseed, rice wheat and amaranth (Wen et al., 2020).

Although most scientists focused their attention on antihypertensive and antioxidant bioactivity of plant-based peptides, there are evidence suggested that BP can also affect the inflammatory processes. A BP derived from rice, chickpea, grains has been demonstrated to have the ability to stimulate the immune system (Santiago-López et al., 2016). According to both *in vitro* and *in vivo* evidences, immunomodulatory peptides are promising compounds for regulating immune responses. This could improve the response to infectious agents and reduce tissue damage (Brandelli et al., 2015). However, an adequate and systematic review of the structures and anti-inflammatory activities of plant-derived BP has still been lacking.

In addition, there are reports that some peptides could have hypocholesterolemic properties, antimicrobial activity, and even capacity to inhibit platelet aggregation and thrombosis (Boparai & Sharma, 2019; Gibbs et al., 2004; Pak et al., 2005).While people become more aware of their health and environment, research on plant-based proteins is becoming more prevalent. The research and technology progress will lead to more available, cheaper, and tastier plant-based protein production, making plant-based proteins a greater option for those who seek to have a nutritious, environmentally friendly, and enjoyable meal.

Sources of plant-based proteins

From a nutritional point of view, the most important aspects of choosing a protein source for hydrolysates, are protein content and composition of amino acids. Plant proteins derived from grains, seeds, legumes, nuts, pulses, and vegetables sometimes could be low or deficient in specific essential amino acids, such as lysine, sulfur-containing amino acids (cysteine and methionine), and threonine (M. Kumar et al., 2022). To overcome this issue, nutrition experts suggest a combination of several plant sources in the diet to obtain appropriate levels of essential amino acids. However, depending on the origin, plants may offer a high-quality protein, they could be rich source of energy, fiber, macro- and micronutrients, as well as bioactive compounds. The protein content of various plant sources is presented in Table 1.1.

Table 1.1 Composition (%) of plants sources used for production of proteins. Adapted from McCance and Widdowson (2015)

Source	*Protein*	*Fat*	*Carbohydrate*	*Moisture*	*Fiber*
Soybeans	35.9	18.6	15.8	8.5	15.7
Chickpea	21.3	5.4	49.6	10.0	10.7
Peas	22.1	2.4	58.2	12.1	6.3
Barley	11.2	61.1	3.1	12.0	17.0
Sorghum	15.6	2.3	72.8	30.0	7.0
Rice	8.1	0.5	83.7	11.3	0.9
Corn	9.4	3.3	73.1	12.2	2.2
Wheat	11.6	2.0	69.9	11.1	8.8
Oats	10.9	8.1	70.7	9.4	7.2
Hemp	25.3	27.0	30.3	7.1	38.1
Alfalfa	35.0	1.3	30.1	15.6	38.0

Among plant sources, soybean has one of the highest protein content, which is 35-40%. It is well balanced in essential amino acids and soybeans are good source of fiber, iron, calcium, zinc, and B vitamins (Lindsay & Claywell, 1998). In addition, soy-based protein products are commonly used for adults and infants who have an allergy to cow milk-based protein product formulas (M. Kumar et al., 2022). Pulses include pea, lupin, chickpea, lentil, bean, and other edible seeds from legume plants are important sources of protein (Day, 2013). Lupin is a leguminous seed that has a protein content around 35-40%. Due to their favorable chemical composition, lupins have recently been used as protein supplements for both humans and animals and widespread availability in many countries (Cabello-Hurtado et al., 2016). In peas the protein content can range from 20 to 30%. Pea is commercially exploited extensively because it can easily be grown all over the world and the hull is simply removed (Pownall et al., 2010). Three forms of pea protein are produced commercially: pea flour, pea protein concentrate, and pea isolate. Wheat is one of the most important food crops in the world, eaten by more than one billion people in various processed forms. Depending on grain variety, wheat contains 8-15% protein. The gluten proteins are the main storage proteins in wheat grains. Gluten is responsible for the formation of a cohesive, visco-elastic proteinaceous network that allows wheat to produce leavened products (Day et al., 2006). Nowadays, there is a variety of commercially produced modified gluten, such as isolated wheat protein, that has enhanced functionality due to further chemical or enzymic treatments of wheat gluten (Day, 2011). Maize contains about 9-12% of protein. Although a small percentage of total corn production is used for human foods, corn is still one of the most important industrial crops, particularly in the USA. About half of the production is used directly as animal feed and a quarter is used for ethanol production (Day, 2013). Furthermore, the cereal grains, barley, rice, and sorghum are also potential sources of plant protein production. Industrial hemp is becoming more popular in the food industry because it is high in both macro- and micro-nutrients. Hemp proteins contain a sufficient level of essential amino acids and are highly

digestible (Shen et al., 2021). It contains from 20 to 30% protein. Alfalfa is one of the recently emerged plant-based protein sources which contains about 15% of proteins in stems and 35% in leaves. Due to high nutritive value and high protein content, alfalfa is widely used in animal and human food. Alfalfa contains many vitamins and minerals such as cooper, folate, iron, magnesium, manganese, vitamin B, vitamin K, vitamin C (Zhang et al., 2017). Alfalfa is considered as a pioneer plant because it can enrich poor soils by producing its own nitrogen fertilizer (Hojilla-Evangelista et al., 2017). Moreover, alfalfa is grown throughout the dry tropical and temperate regions of the world (Kang et al., 2011). In this study the alfalfa source is chosen as a protein source for the experiments of plant-based protein hydrolysis.

1.4 Conventional and novel methods for generation of protein hydrolysates

The plants are good source of proteins, however, most of these sources have limited applications for protein processing due to their poor aqueous solubility and structural complexity (Warnakulasuriya & Nickerson, 2018). Plan proteins are often embedded with fiber which consists of cellulose, hemicellulose, and lignin. These compounds reduce the ability of digestive enzymes to reach the proteins. To improve the functionality and availability of plant-based proteins, hydrolysis process could be implemented. Protein hydrolysate is the most nutritious form of protein source as contains a mixture of short chain peptides and free amino acids, which are more readily absorbed by the body. The processing principle of the plant-based protein hydrolysis is demonstrated by the workflow scheme in Fig. 1.4.

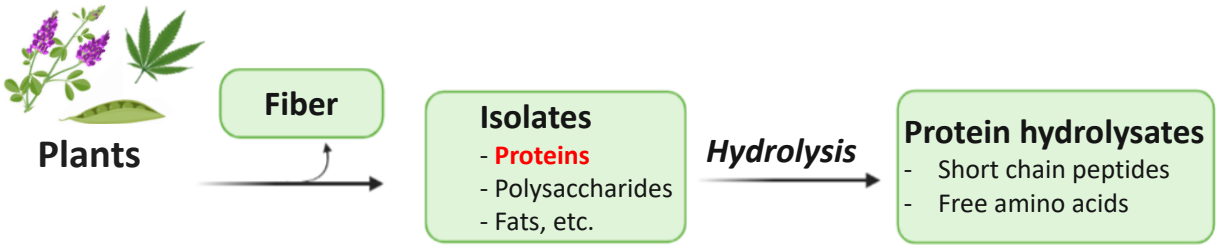


Fig. 1.4 Workflow of plant-based protein hydrolysis

The first step involves a removal of fiber compounds to increase accessibility of proteins. For example, enzymes that targets the cell wall integrity could be used to degrade indigestible fiber components. The further step includes precipitation and ultrafiltration methods, or a combination of both, during which isolates are extracted from plants. As mentioned before, isolates could contain some other macronutrients such as polysaccharides, fats, etc., but mainly it is partially purified form of plant proteins. After the latter step, isolates can be further hydrolyzed by the selected hydrolysis method. As an alternative to isolates, plant meal or even isolated pure proteins could be used for the hydrolysis.

Methods for protein hydrolysis process

Conventional protein hydrolysis techniques mainly involve chemical methods. Chemical hydrolysis is conducted using acid or alkali at high temperatures. Hazardous conditions during chemical hydrolysis process could destroy some amino acids or even generate toxic compounds. During chemical hydrolysis, complete digestion of protein into free amino acids can be done at high temperatures and long reaction time. Lower temperatures and shorter reaction time are often used for generation of short chain peptides. Although alkaline hydrolysis is a common method for producing foaming agents, it is not used in the food industry, acid hydrolysis is more frequent (Hou et al., 2017). Despite the fact that chemical hydrolysis method is a low cost, the process results in the destruction of tryptophan, the loss of methionine, and the conversion of glutamine into glutamate and asparagine into aspartate, making this method unfavorable in food industry (Pasupuleti et al., 2008). Oppositely, enzymatic hydrolysis is often preferred to chemical hydrolysis due to several reasons (**Fig. 1.5**).

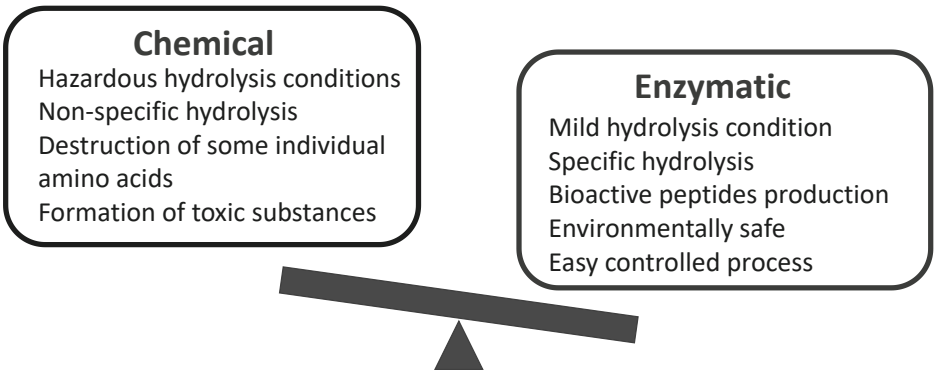


Fig. 1.5 Comparison of chemical and enzymatic hydrolysis processes

The enzymatic reactions are performed by proteases under mild conditions. Typically, it requires medium pH and gentle temperature. It is easy controllable process due to specificity of enzymes. Usually it avoids side reactions, does not decrease the nutritional value and retain the quality of proteins (Hou et al., 2017). In addition, it is environmentally safe process, where the composition of the final product can be controlled by selecting of specific enzymes and reaction conditions.

Fermentation is another technology for the production of protein hydrolysates. During fermentation, microorganisms produce enzymes that are released into extracellular medium where proteins are hydrolyzed. Different starter cultures could be used for the fermentation, but lactic acid bacteria (LAB) is the most common (Rizzello et al., 2016). LAB strains have a high potential in fermentation because each species could exhibit different proteases content, leading to a large variety of proteolytic activities. Diversity of hydrolysates with different types of short chain peptides and different bioactivity is a major interest in fermentation. However, it is difficult to produce peptides through fermentation on an industrial scale due to a low yield, inability to control and predict the composition of final product and contamination risk (Raveschot et al., 2018). Contamination is a major concern as it can lead to off-flavors and off-odors and can also cause food safety concerns.

Precision fermentation is one of the emerging technologies which could solve mentioned issues. It uses genetically engineered microorganisms to produce desired compounds, such as proteins, peptides, amino acids, with the high yields and purity. This type of precise control enables faster, more efficient, and more consistent production of a variety of products, including peptides (Teng et al., 2021).

In conclusion, enzymatic hydrolysis is foreseen as a prominent method in comparison with fermentation or chemical hydrolysis process due to the absence of toxic, chemical or microbial contaminants. It is effective and controllable process for producing functional and nutritious food, as well as generating bioactive peptides.

Improvement of enzymatic protein hydrolysis process

To improve the degree of hydrolysis and the yield of short chain peptides and amino acids, novel protein hydrolysis technologies are being explored, especially when protein isolates are used. The main principle of this novel approach is a combination of methods which relies on physical processes with enzymatic hydrolysis. For example, *ultrasound-mediated method* is a non-thermal method for producing peptides by passing ultrasonic waves through a protein mixture (Ulug et al., 2021). Ultrasound is an acoustic wave with a frequency greater than 20 kHz which needs a medium to propagate (Mason & Lorimer, 2002). High-intensity ultrasound disrupts protein aggregates, changes protein form, which could in turn increase the degree of hydrolysis and bioactivity. This is because the ultrasound can affect hydrogen bonds and hydrophobic interactions, can generate cavitation, and can disrupt the quaternary and/or tertiary structure of proteins. As a result, during enzymatic hydrolysis, the enzyme may be able to access more hydrolysis sites after such pretreatment (Ozuna et al., 2015) *High hydrostatic pressure (HHP) processing* is other approach that has the potential to improve the efficiency of enzymatic hydrolysis. This technology uses isostatic pressures between 100 and 1000 MPa, with or without heat treatment. The HHP process is a batch system in which water transmits pressure as a medium, which has been used in the food industry to enhance the microbial safety, nutritional, and functional properties of food products (Naderi et al., 2017). HHP processing can improve the efficiency of enzymatic hydrolysis by causing protein unfolding and increased interaction between enzymes and proteins (B. P. Singh et al., 2022). The food industry is also trying to employ *microwave-assisted technology*, which uses microwave energy to produce molecular motion via ionic conduction and dipole rotation. It is useful technology for generation of hydrolysates in conjunction with protease enzymes. Microwaves contribute to the enhanced protein hydrolysis by exposing the cleavage sites of proteins which resulted in rapid protein disintegration (Zhong et al., 2005). This method has been widely used to extract bioactive compounds from plant materials in the food industry (B. P. Singh et al., 2022). *Pulsed electric field (PEF) processing* is

another potential method for producing peptides from food protein. This technology is a non-thermal, has low energy consumption, and is quick. The principle of pulsed electric field involves the application of short, high-voltage pulses to liquid or semi-liquid foods placed between a pair of electrodes. The PEF enhances the production of peptides by unfolding and denaturing proteins by disrupting noncovalent bonds in quaternary structures, such as hydrogen bonds and hydrophobic interactions (Ulug et al., 2021).

Technology innovation plays an important role in unlocking more opportunities for applying plant-based protein hydrolysates in industry. As there is a projected growing tendency in production of alternative proteins, there is a high need to develop effective strategies to improve the nutritional value and functional properties of plant proteins.

Identification of peptides from plant-based protein hydrolysates

The identification of peptides from plant-based protein hydrolysates is an important area of research. It can be challenging to detect all peptides due to the fact that hydrolysates are being composed of a wide variety of peptides with varying lengths and concentrations. Foremost, hydrolysates are frequently fractionated and purified, using various methods such as ultrafiltration, high-performance liquid chromatography (HPLC), ion-exchange HPLC, etc. (B. P. Singh et al., 2022). After that, various methods for peptide identification are used. X-ray crystallography and nuclear magnetic resonance (NMR) have played a significant role in elucidating the structures of pure peptides (Krishnan & Rupp, 2012). X-ray crystallography studies require a protein that can crystallize, whereas NMR is suitable for macromolecules that are in solution. However, many proteins or peptides cannot be analyzed with these high-resolution techniques because they are not soluble, are unstable or cannot form crystals. Other techniques for the characterization of proteins and peptides include electron microscopy, fluorescence resonance energy transfer, and chemical cross-linking (Patil et al., 2020). Mass spectrometry is the major tool used in analyzing peptide mixtures and measuring the mass-to-charge ratio (m/z) of one or more molecules present in a sample. Amino acid sequencing in mass spectrometry can be performed using two methods: top-down sequencing and down-top sequencing. The softwares that use an amino acid sequence algorithm are widely available for protein and peptide identification (Wysocki et al., 2005). However, each method has advantages and disadvantages and should be carefully evaluated before the use.

Identification of bioactive peptides

In the production of protein hydrolysates, some part of the formed short chain peptides could exhibit specific bioactivity. However, challenges for the production and commercialization of bioactive peptides (BP) include the identification technique shortage. The classical *in vitro* and novel *in silico* approaches of peptide identification are presented in Fig. 1.6.

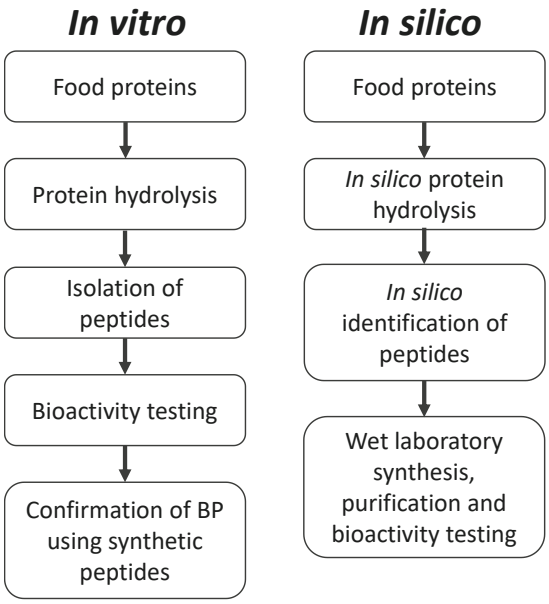


Fig. 1.6 Classical and novel approaches for BP identification

The classical approach is based on protein hydrolysis which is performed *in vitro*, followed by purification of peptides by various methods such as ultrafiltration, chromatographic purification, etc. Purified peptides are further tested for bioactivity. Specific *in vivo* and *in vitro* techniques for each type of bioactivity could be used, however, *in vivo* studies of bioactivity are not conducted usually due to high costs and difficulties in implementation. Methods for bioactivity assessment of peptides are presented in Fig 1.7.

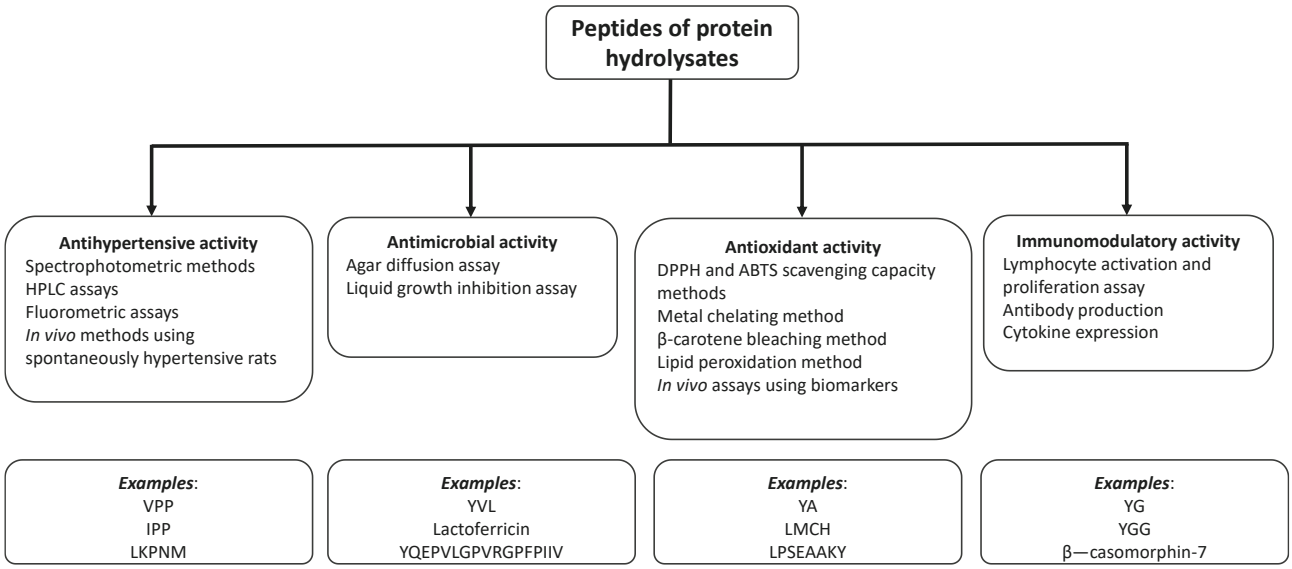


Fig. 1.7 Methods for bioactivity assessment of peptides and examples of bioactive peptides

The most used methods for antihypertensive determination are based on spectrophotometric, HPLC and fluorometric assays. For example, spectrophotometric assay is conducted using ACE-specific substrate hippuryl-l-histidyl-l-leucine (HHL) (Li et al., 2005). *In vivo* assays are performed using spontaneously hypertensive rats by administrating of peptides (Nardo et al., 2020). For

antimicrobial evaluation, agar diffusion technique is highly used for assessment of antibacterial, antiviral, or antifungal peptides. In liquid growth inhibition method, the antimicrobial activity is determined by inoculating bacteria into 96-well microtiter plates with different concentrations of antimicrobial agent (Wiegand et al., 2008). To assess antioxidant properties of peptides, there are plenty of methods such as DPPH and ABTS scavenging capacity method, metal chelating method, β-carotene bleaching method, lipid peroxidation and etc. (Xing et al., 2019). *In vivo* assays of antioxidant peptides are conducted using biomarkers (Carrasco-Castilla et al., 2012) Immunomodulatory activity is assessed by lymphocyte activation and proliferation, antibody production and cytokine expression. Still, it is difficult to evaluate such bioactivity since a number of factors are responsible for immunomodulatory effects.

After the fractions of peptides shown specific bioactivity, peptides are sequenced to determine the amino acid sequence. Once the structure is known, confirmation of bioactivity is conducted using chemically synthesized identical peptides and performing the same bioactivity tests (Agyei et al., 2016). This classical approach has several drawbacks: i) it generates low yields of peptides; ii) limited number of peptide species can be investigated at the same time; iii) it is laborious, expensive, and time-consuming method (Udenigwe, 2014).

The novel approach is based on protein hydrolysis and prediction of peptides *in silico*. This method provides an information about predictable bioactive peptides from plant-based proteins prior to wet-laboratory experiments. The method is performed by few steps. Firstly, in protein databases you could select desired protein with known amino acid sequence. Then, these proteins are hydrolyzed *in silico* using appropriate proteolytic enzymes. It can assist in selecting the most suitable enzyme for a given protein. Such computational hydrolysis generates peptides which further are characterized to identify structural properties. Using various bioinformatic tools and databases peptides are identified and potential bioactivity is evaluated. The resulted information is followed by validation in wet laboratory experiments. Launched in 2003, BIOPEP-UWM is one of mostly mentioned databases in the literature, which is used for *in silico* study of bioactive peptides. This database is designed of interlinking three databases of protein sequence, bioactive peptides, and proteolytic enzyme (Minkiewicz et al., 2019). *In silico* method enables the simultaneous identification of bioactive peptides from a variety of proteins and proteases. Also, it is quick, relatively cheaper, and less laborious method which is used for fast initial screening of potential sources for bioactive peptides. Combining classical approach with bioinformatics leads to comprehensive search for bioactive peptides. While *in silico* methods become more popular, large and reliable databases are required with adequate amount of experimental evidence.

1.5 Proteases for enzymatic hydrolysis

Application of proteases

Enzymatic hydrolysis is performed by enzymes called proteases. Proteases catalyzes the cleavage of peptide bonds between amino acids. Approximately 65% of all enzymes in global market accounts to proteases (Gimenes et al., 2019). The commercial application of proteases includes various industry sectors (Fig.1.8).

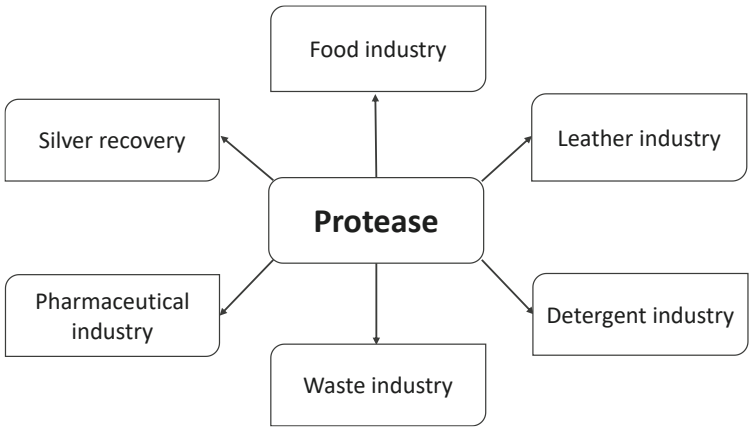


Fig.1.8 Application of proteases in different sectors

In food industry, proteases have a significant function in producing high quality and nutritious food products. Proteases can increase the shelf life of products, which is crucial for food industry (V. Kumar et al., 2019). Production of candies and milk products require a complete processing that is catalyzed by series of proteases. Beverage industry uses proteases to improve flavor of fruit juice or soft drinks. Wheat flour is a major ingredient used in baking, containing an insoluble protein known as gluten which affects the properties of the dough. Protease can break down the gluten and improve the quality of the product in terms of color, softness, and texture (Souza et al., 2015).

In leather industry, the traditional processing of leather involves a lot of toxic chemical compounds which contribute to the pollution of environment. Leather mainly consists of collagen, which can be broken down by proteases. Seeking to find alternatives to traditional processing, the use of proteases was one of the solutions which lead to improved leather quality and the decreased pollution (Sharma et al., 2019). Alkaline proteases are mostly used in leather processing.

In detergent industry, protease enzymes have extensive application due to ability to remove a great variety of stains. The alkaline and thermostable enzyme has major applications in the detergent industry. Several hydrolytic enzymes can be used in combination, for example, the use of proteases and amylases in laundry detergents are very common (V. Kumar et al., 2019).

In waste treatment, alkaline proteases exhibit the capacity to degrade waste from food-processing industries and household activities, thereby playing a significant role in waste management (Sharma et al., 2019). For example, in poultry industry, feathers contain a keratin

structure which is a rich source of proteins. Microbial proteases are used in chicken feather processing, where they are converted into highly digestible animal feed (Khardenavis et al., 2009).

In pharmaceutical industry, a wide variety of proteases are used as effective therapeutic agents. For example, proteases have been used as an aid for digestive disorders to correct certain lytic enzymes deficiency (Meghwanshi et al., 2020). Some types of proteases are used in combination with broad-spectrum antibiotics in treatment of wounds. Also, proteases can act as anticancer, anti-inflammatory and clot-buster agents (Srilakshmi et al., 2014).

Silver recovery. Proteases are also used in silver recovery of utilized X-ray films which contains about 1.5-2% silver in its gelatin layer. During photography, silver is not demolished and thus can be recovered and reused. The conventional silver recovery methods are based on burning the films which causes environmental pollution. An alternative to burning, the use of proteases provides a safe and cost-effective way of extracting silver from X-ray films by removing the gelatin layer and recovering the silver (R. Singh et al., 2016).

Classification of proteases

Proteases constitute a very large and complex group of enzymes. They differ among themselves in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature optima, and stability profiles (Gimenes et al., 2019). Generally, based on the pH optima, proteases are referred to as acidic (pH 2.0-3.5), neutral (pH 6.0- 7.5), or alkaline (pH 8.0-11.0) proteases.

Proteases, depending on the catalytic site, are classified as endopeptidases or exopeptidases (**Fig. 1.9**).

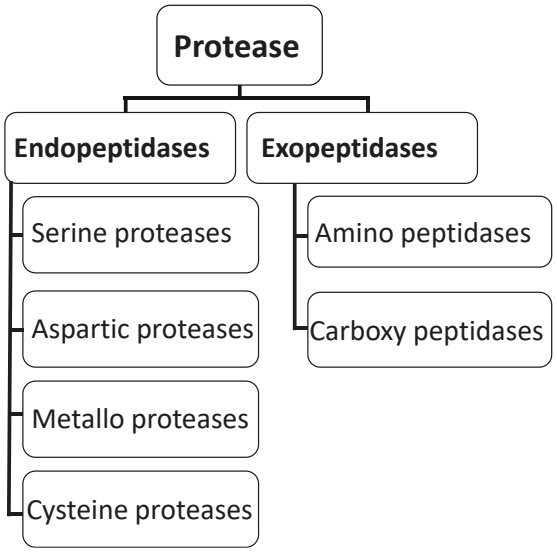


Fig. 1.9 Classification of proteases

Endopeptidases act on the interior of polypeptide chains. Based on the functional group presented in the enzyme active site, endopeptidases are further classified into four groups: serine proteases, aspartic proteases, metalloproteases and cysteine proteases. Exopeptidases, based on the

site of action at the carboxy or amino terminus of the substrate, are further divided into amino peptidases and carboxy peptidases groups.

Sources of proteases

Proteolytic enzymes in all living organisms plays an essential role in various physiological processes including zymogen activation by proteolysis process, blood coagulation, protein catabolism, inflammation processes, cell growth, tissue arrangement and morphogenesis (Rao et al., 1998; Souza et al., 2015). Proteases are produced by plants, animals, and microorganisms. Historically, enzymes from all these sources have been used, however nowadays, to meet the huge demand of industrial scale, microorganisms are the main source of the proteolytic enzymes. Approximately two-thirds of the commercial proteases used around the world are derived from microorganisms (Razzaq et al., 2019). Microbial proteases have become favored for biotechnological applications due to their high yield, short production time, small space requirement, high potential for genetic manipulation, and cost-effectiveness (Ali et al., 2016). Besides, a lot of recombinant enzymes could be synthesized by microorganisms. Most of the microbial proteases are secreted out into the liquid broth where extraction and purification of the enzyme are simple. Among bacterial sources, *Bacillus* and *Streptomyces* genera are the most used at the industrial level. Fungal proteases have lower reaction rate and heat stability compared to bacterial origin proteases; nevertheless, they represent wider variety of proteases with broad substrate specificity (Jisha et al., 2013). The genera of *Aspergillus, Humicola*, *Mucor*, *Penicillum*, *Rhizopus* also represent a great variety of proteases (Singh et al., 2016).

The most important industrial proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin, and rennin. Trypsin is the main intestinal digestive enzyme present widely in vertebrates. Usually, in industry, trypsin is used for preparation of growing medium for microorganisms. Chymotrypsin is extracted from the pancreatic juice of animals. It is an expensive enzyme used only for diagnostic and analytical purposes. Pepsin exists in stomachs of almost all vertebrates. It is an acidic protease which had been used in laundry detergents in early twentieth century, and now it is replaced by alkaline microbial proteases which are less degradable by detergents, at alkaline conditions and at the high temperatures (Jisha et al., 2013). Rennet is a pepsin-like protease, which is produced as inactive form in the stomach of nursing mammals. It is converted to active rennin by the action of pepsin. Rennet from calf had a huge demand in the dairy industry for production of milk products. All these animal-origin proteases were being obtained from slaughtered cattle, however, to meet the demand of protease production from industry, recombinant versions are being produced.

Plants proteases are also used in industry. Plant proteases can be obtained from latex, fruits, leave or stem of plant. The most extensively studied plant-origin proteases are papain, ficin and

bromelain extracted from papaya, fig, and pineapple, respectively. Plant proteases represent 5% of the global sales of proteases (Mazorra-Manzano et al., 2018). Application of these proteases include meat tenderization, milk coagulation, brewing, baking, production of hydrolysates, viral treatment (Kumar et al., 2019; Singh et al., 2016). The new emerging plant protease sources are ginger, melon and kiwifruit, that could produce serine proteases. Despite the fact that plants have gained an increasing attention due to their potential to produce various protease, extraction of plants proteases is a time-consuming process. Additionally, land area and climate conditions are the main factors also for cultivation of plants.

In conclusion, proteases can be obtained from various sources of organisms; however, microorganisms have more advantages over plants and animals: higher yields of production, grow faster, easy to manipulate genetically, require less space, and much simpler purification.

Recombinant proteases and their expression systems

A lot of commercial proteases are recombinant and are produced applying host expression systems. These systems involve the cloning of the gene of interest into a host organism and it allows the large-scale production of enzymes. Recombinant proteases production is beneficial for various industrial applications. Additionally, genetic engineering leads to produce recombinant proteases with improved activity, enhanced stability, and a wider substrate specificity.

The mostly used bacterial expression systems include *Escherichia coli*, *Bacillus subtilis* and *Lactococcus lactis*. Bacterial expression systems have several advantages including rapid and high-level of expression, simple and inexpensive nutritional components for the media, fast and easy transformation process (Karbalaei et al., 2020). *E. coli* is one of the most important cells that has been widely used for cloning of recombinant DNA and subsequently, for the production of heterologous proteins (Baneyx, 1999). *B.subtilis* and *L.lactis* are classified as GRAS (*generally recognized as safe*) organisms, so they are safe expression systems that could be applied in pharmaceutical or food industry. However, bacterial expression systems have some limitations such as intracellular aggregation and misfolding of recombinant protein, lack of posttranslational modification and protein degradation due to host proteases (Rosano & Ceccarelli, 2014).

Yeasts are another expression system widely used for heterologous proteases production. Compared with bacteria, yeasts have significant advantages including growth speed, posttranslational modification, secretory expression, and easy genetic manipulation. Species established in industrial production are *Saccaromyces cerevisiae*, *Kluveromyces lactis*, *Pichia pastoris* and *Hansenula polymorpha*. *P.pastoris* is the most frequently used yeast species due to its high production yield, low levels of native proteins and simplified protein purification (Sun et al., 2018). A variety of proteases have been successfully expressed in *P. pastoris*, including serine protease from *Trichoderma koningii*

(Shu et al., 2016), alkaline protease from *Aspergillus oryzae* (Guo & Ma, 2008) and neutral protease from *Aspergillus oryze* (Ke et al., 2012). Also, a lot of promoters can be used for the recombinant protein production in *P.pastoris* expression system.

Serratopeptidase

Serratopeptidase, also known as serralysin, serrapeptase or serratiopeptidase, is an alkaline metalloprotease. Serratopeptidase is made up of 470 amino acids (a.a.) and was originally isolated in the late 1960s from the opportunistic pathogen *Serratia marcescens* microorganism which was present in the silkworm intestines. Serratopeptidase is responsible for the dissolution of cocoon in emerging moth. Serratopeptidase shows the highest activity at pH 9.0 with a temperature 40 ˚C and gets deactivated within 15 min at 55 ˚C (Dhiman & Purohit, 2022). As many metallopeptidases, metal ions are required for serratopeptidase catalytic site. It binds one zinc and seven calcium ions to the active site. Like other proteases which have metal ions in the active site, serratopeptidase activity is inhibited by EDTA which is chelating agent (Chander et al., 2021). Compared to other proteases, serratopeptidase has a broad substrate specificity. Generally, proteases have a few cleavage sites, while serratopeptides cleave the peptide linkages of Asn-Gln, Cys-Gly, Arg-Gly, Tyr-Tyr, His-Leu, Gly-Ala, Ala-Leu, Tyr-leu, Gly-Gly, Phen-Tyr, and Tyr-Thr amino acids. Most of the studies of serratopeptidase in the literature are dedicated for enzyme investigation in pharmaceutical field, however, there are lack of reports of application in other industries. The main properties of serratopeptidase in therapeutic applications are present in Fig. 1.10.

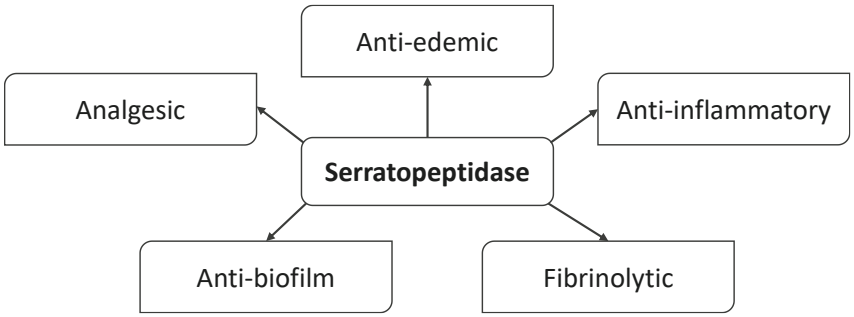


Fig. 1.10 The main properties of serratopeptidase in therapeutic applications.

Anti-inflammatory effect, exhibited by serratopeptidase, is one of the features that is of interest in pharmaceutical sector. Clinical studies have shown that serratopeptidase is helpful in reducing swelling and edema, metabolizing scar tissues (Chappi D. et al., 2015). Also, it has fibrinolytic properties and can hydrolyze dead tissue, blood clots, cysts, and arterial plaques. Serratopeptidase can be used together with antibiotics to enhance their efficacy by inhibition of bacterial biofilm formation. In addition, the ability of serratiopeptidase to hydrolyze bradykinin, histamine, and serotonin contributes to its analgesic activity (Jadhav et al., 2020).

A broad specificity of serratopeptidase is important for industrial applications. Since the origin source of enzymes are opportunistic pathogen, suitable recombinant expression system is needed for

serratopeptidase production. According to the literature, *E. coli* expression system is not suitable for serratopeptidase production as it is toxic for the *E. coli* cells. However, there is a study whereas the expression was carried out in the modified *E. coli* strains which was design for toxic protein expression (Srivastava et al., 2019). In this study, serratopeptidase was overexpressed in inclusion bodies and required refolding; nonetheless, the purified protein was enzymatically inactive. To achieve the production of active serratopeptidase, yeast expression system was investigated. Serratopeptidase gene was cloned into *Pichia pastoris*. An active form of enzyme was secreted into the medium (Kaviyarasi et. al, 2016). It was concluded that *P. pastoris* is suitable and effective host system to secret active serratopeptidase. In addition, the capacity of *P. pastoris* to secrete into the medium makes it a suitable and promising system for production of serratopeptidase.

In this work, serratopeptidase will be used for investigation of plant-based protein hydrolysis process. Serratopeptidase have never been applied in protein hydrolysis but it has a broad spectrum of cleavage sites, making potential for effective hydrolysis of plant proteins. Also, the origin of microorganism isolated from plants makes it promising enzyme for plant-based protein hydrolysis.

2. MATERIALS AND METHODS

2.1 Devices and tools

Table 2.1 The list of equipment which was used during the investigation.

Analytical balance	Kern ABJ
ATR-FTIR spectrometer	Bruker ALPHA
Balance	Kern E6
Biological safety cabinet	Flow Fast H
Centrifuges	Eppendorf 54 (rotor r = 84 mm) Labnet Prism R (rotor r = 84 mm) Labnet Prism Mini
Electroporator	BioRad
Horizontal electrophoresis system	BioRad
Spectrophotometer	Nanodrop 2000; Spekol 2000
Thermocycler	BioRad iCycler
Thermostat	BINDERE 28 BiosanES20
Vortexes	Vibramax 100
Lab shakers	Stuart Orbital Incubator Biosan
pH meter	Mettler Toledo
Transilluminator	Biostep
Laboratory water bath	Thermo Haake P5
Magnetic stirrer	Biosan MMS-3000 Fisherbrand isotemp IKA RT 10
Microwave	Daewoo Scarlett

2.2 Materials and solutions

2.2.1 Microorganisms and vectors

Microorganisms: *Escherichia coli* DH5α *recA, endA, Pichia pastoris* GS115 strain.

Vectors: cloning vector pUC18, expression vector Pic9K (Invitrogen), BB3aZ_14* (GoldenPics plasmid toolkit)

2.2.2 Primers

Table 2.2 Primers and their DNA sequences (Invitrogen).

P652	5‘ CCTACTTGACAGCAATATATAAACAGAAGG 3’
P653	5’ GGAGCAAGCTCGTACGAGAAG 3’
P705	5’ CAAGCAGCAGATTACGCGCAG 3’
P706	5’ GAAGCTATGGTGTGTGGTACCGATC 3’
P708	5‘ AGCAGGAATTCCAATCTACTAAAAAGGCAATTGAAA 3‘
P709	5‘ GACATGCGGCCGCTTAGTGGTGATGGTGATGATGCACGATAAAGTCCGTGGCGAC 3‘
P710	5‘ AGCAGGAATTCGCGAGCTCCGCTTACAATG 3’
P711	5’ GACATGCGGCCGCTTACACGATAAAGTCCGTGGC 3’
P716	5‘ TTGAAGACGCCATGCAATCTACTAAAAAGGCAAT 3‘
P717	5‘ GCGAAGACGGAAGCTTAGTGGTGATGGTGATGAT 3‘

P718	5‘ TTGAAGACGCCATGAGATTTCCTTCAATTTTTACTG 3‘
P719	5‘ GGCCAGCGAGGACTACGGC 3’
P720	5‘ GCCGTAGTCCTCGCTGGCC 3’
P721	5‘ ACACCTTTGTGTTCTCTGCGG 3’
P722	5‘ CCGCAGAGAACACAAAGGTGT 3’

2.2.3 Reagents

“Acros Organics”: agar, ammonium sulfate (99 %), D-sorbitol (97 %), calcium chloride (96 %), sodium hydroxide (99 %).

“BioRad”: tris(hydroxymethyl)aminomethane (Tris) (≥ 99.8 %).

“Fisher Bioreagents”: LB medium (10 g/L peptone from casein, 10 g/L NaCl and 5 g/L yeast extract) molecular genetics grade.

“Merck & Co., Inc.”: yeast extract, hydrogen chloride, magnesium chloride hexahydrate (≥ 95 %), agarose, ampicillin (sodium salt) (≥ 90 %), D(+)gliucose monohydrate, sodium chloride (≥ 99.5 %),

“Roth”: etidium bromide.

“Sigma-Aldrich”: N,N-dimethylated casein; bovine serum albumin (BSA).

“Thermo Fisher Scientific”: 6×DNA Gel Loading Dye, 1 Kb Plus DNA Ladder, GeneRuler 100 bp DNA Ladder, Carrier DNA, TopVision Agarose, DTT, ethylenediaminetetraacetic acid (EDTA) (≥ 99 %), DMSO (100 %), Yeast Nitrogen Base (YNB) (without amino acids and ammonium sulfate), peptone, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (≥ 99 %), Di-Potassium hydrogen phosphate.

“Serva”: Potassium chloride.

“Oxoid”: Skimmed milk.

2.2.4 Kits and enzymes

Kits (Thermo Fisher Scientific): “GeneJET Plasmid Miniprep Kit”, “GeneJET PCR Purification Kit”, “GeneJET Gel Extraction Kit”.

PCR Master Mixes (Thermo Fisher Scientific): “Phusion Hot Start II High-Fidelity PCR Master Mix (2×)”, “DreamTaq Green PCR Master Mix (2×)”.

Restriction endonucleases (10 U/µL) (Thermo Fisher Scientific): *Eco*RI, *Not*I, *Sac*I, *Dpn*I, *Aat*I, *Sal*I, *Xba*I, *Bpi*I, *Pag*I, *Mls*I, *Bsp*1407I, *Sgs*I, *Bsh*TI.

Reaction buffers for restriction endonucleases (Thermo Fisher Scientific): 10×Orange Buffer, 10× BamHI Buffer, 10× Tango Buffer, 10× FastDigest Buffer, 10×FastDigest Green Buffer.

Enzymes: *Vilzim PRO AD3000* ”Biorro”; *Vilzim PRO NL200* ”Biorro”; *Vilzim PRO AL600* ”Biorro”.

2.2.5 BSA solution for calibration of ATR-FTIR spectroscopy method

10 mg/ml bovine serum albumin (BSA) standard: 20 mg of BSA are dissolved in 10 ml 200 ml of distilled H_2O.

2.2.6 Solutions for protease activity measurement

0.4% N,N-dimethylated casein (DMC) substrate solution: 0.2 g DMC is dissolved in 10 ml of boiled distilled H_2O. In parallel, 1.62 g of $Na_2B_4O_7 \times 10H_2O$ and 0.83 g of $NaH_2PO_4 \times 2H_2O$ are dissolved in 15 ml of distilled H_2O. pH was adjusted to 8.0 with NaOH. Both solutions are mixed and diluted to final volume of 50 ml with distilled H_2O. The obtained solution is stored at 4°C.

0.1 % 2,4,6-Trinitrobenzene sulfonic acid (TNBS) solution: 0.2 ml of 5% TNBS is diluted to final volume of 10 ml with distilled H_2O.

BB9 buffer pH 9.0: 2.8 g of KCl, 5 g of Na_2SO_3 and 0.8 g of $B(OH)_3$ are dissolved in 200 ml of distilled H_2O. The pH is adjust to 9.0 with NaOH and obtained solution is diluted to final volume of 250 ml with distilled H_2O.

2.2.7 Solutions for hydrolysis reaction

0.01 M potassium phosphate buffer: 0.02 g of KH_2PO_3 and 0.4 g K_2HPO_3 are dissolved in 200 ml of distilled H_2O. Then, using H_3PO_4 or NaOH pH is adjusted to appropriate value. The final volume is adjusted to 100 ml.

10 mg/ml sodium nitrite: 20 mg of sodium nitrite is dissolved in 2 ml of distilled H_2O.

2.2.8 Cultivation media for *E. coli*

LB (Luria-Bertani) agar medium: 5 g of LB and 3 g of agar are dissolved in 200 ml of distilled H_2O and autoclaved. For selection of transformants, additionally 100 μg/ml of ampicillin was added into medium.

LB liquid medium: 2.5 g of LB is dissolved in 100 ml of distilled H_2O and autoclaved. For selection of transformants, additionally 100 μg/ml of ampicillin is added into medium.

100 mg/ml ampicillin solution: 0.1 g of ampicillin is dissolved in 1 mL of distilled H_2O.

2.2.9 Solutions for of competent *E. coli* cells preparation and transformation

NaCl solution: 0.29 g of NaCl, 0.05 g of $MgCl_2 \times 6H_2O$ and 0.03 g of Tris are dissolved in 40 ml of distilled H_2O. Using HCl the pH is adjusted to 8.0 and the volume is brought to 50 ml. Then the solution is autoclaved.

$CaCl_2$ solution: 0.56 g of $CaCl_2$, 0.05 g of $MgCl_2 \times 6H_2O$ and 0.03 g of Tris are dissolved in 40 ml of distilled H_2O. Using HCl the pH is adjusted to 8.0 and the volume is brought to 50 ml. Then the solution is autoclaved.

2.2.10 Cultivation media for *P. pastoris*

YEPD medium: 2 g of yeast extract, 4 g of peptone and 4 g of glucose are dissolved in 200 ml of distilled H_2O and autoclaved at 0.8/30.

YEPD agar medium: 2 g of yeast extract, 4 g of peptone, 4 g of glucose, and 3 g of agar are dissolved in 200 ml of distilled H_2O and autoclaved at 0.8/30. For selection of transformants, additionally appropriate concentration of antibiotic is added into medium.

BMMY medium: 1 g of yeast extract and 2 g peptone are dissolved in 79.3 ml of distilled H_2O and autoclaved. Then, 10 ml of 1M pH 6.0 potassium phosphate buffer, 10 ml of 10X YNB, 0,2 ml of 500X Biotin and 0,5 ml of methanol is added.

BMGY medium: 1 g of yeast extract and 2 g peptone are dissolved in 79.3 ml of distilled H_2O and autoclaved. Then, 10 ml of 1M pH 6.0 potassium phosphate buffer, 10 ml of 10X YNB, 0,2 ml of 500X Biotin and 1 ml of glycerol is added.

10X YNB solution: 3,4 g of YNB (without ammonium sulfate and amino acids) and 10 g of ammonium sulfate are dissolved in 100ml distilled H_2O. Then, the solution is filter sterilized and stored at 4°C.

500X Biotin solution: 20 mg biotin is dissolved in 100 ml of distilled H_2O and filter sterilized. The stock solution is stored at 4°C.

1 M pH 6.0 potassium phosphate buffer: 11.88 g of KH_2PO_3 and 6.56 g K_2HPO_3 are dissolved in 90 ml of distilled H_2O. Then, using H_3PO_4 pH is adjusted to 6.0 and final volume is adjusted to 100 ml. The solution is autoclaved.

2.2.11 Solutions for electrocompetent *P.pastoris* cells preparation and electroporation

40 mM HEPES/YEPD medium: 4 ml of 1 M HEPES is added to 48 ml of YEPD medium.

1 M Sorbitol: 18.22 g of sorbitol is dissolved in 70 ml of distilled H_2O, the final volume is adjusted to 100 ml and autoclaved.

50 mg/ml geneticin solution: 0.1 g of geneticin is dissolved in 2 ml of distilled H_2O.

YPD medium: 2 g of yeast extract, 4 g of peptone and 4 g of glucose are dissolved in 200 ml of distilled H_2O and autoclaved.

1 M HEPES solution, pH 8.0: 11.9 g of HEPES is dissolved in 30 ml of distilled H_2O. Using 1M NaOH the pH is adjusted to 8.0 and volume is brought to 50 ml. Then, solution is sterilized using 0.22 μm filter.

1M DTT solution: 3,85 g of DTT is dissolved in 22 ml of distilled H_2O. Using 1M NaOH the pH is adjusted to 8.0 and sterilized using 0.22 μm filter.

2.2.12 Solutions for SDS-PAGE electrophoresis

10 % Ammonium persulphate (APS) solution: 1 g of APS is dissolved in 10 ml of distilled H_2O.

10 % Sodium dodecyl sulfate (SDS) solution: 5 g of SDS is dissolved in 40 of distilled H_2O and then the volume is brought to 50 ml. The solution is stored at 20-22°C temperature.

1.5 M Tris-HCl buffer, pH 8.8: 18.15 g of Tris is dissolved in 70 ml of distilled H_2O. Using HCl the pH is adjusted to 8.8. The volume is brought to 100 ml. The solution is stored at 4°C, in a dark glass bottle.

1 M Tris-HCl buffer, pH 6.8: 12.10 g of Tris is dissolved in 50 ml of distilled H_2O. Using HCl the pH is adjusted to 6.8. The volume is brought to 100 ml. The solution is stored at 4°C, in a dark glass bottle.

30% acrylamide (AA) and bisacrylamide (BAA) solution: 58.4 g of AA and 1.6 of BAA are dissolved in 100 ml of distilled H_2O. The volume is brought to 200 ml and filtered. The solution is stored at 4°C, in a dark glass bottle.

10× concentrated stock of SDS-PAGE buffer: 15 g of Tris, 72 g glycine, and 5 g of SDS are dissolved in 380 of distilled H_2O. 1×SDS-PAGE buffer is used for electrophoresis.

2.2.13 Solutions for DNA electrophoresis

0.5 M EDTA, pH 8.0: 29.2 g of EDTA is dissolved in 180 ml of distilled H_2O. pH of the solution is adjusted to pH 8.0. The volume of solution is brought to 200 ml and autoclaved.

10×TAE buffer: 24,3 g of Tris, 5.7 ml of glacial acetic acid and 10 ml of 0.5 M EDTA (pH 8.0) are dissolved in 500 ml of distilled H_2O. 1×TAE buffer is used for electrophoresis.

1 mg/mL etidium bromide solution: 0.1 g of etidium bromide is dissolved in 100 ml of distilled H_2O.

2.3 Methods

2.3.1 ATR-FTIR spectroscopy method for protein concentration measurement

The infrared spectrum of protein exhibits three major characteristic bands (*Amide I, Amide A, Amide II*) in the range 4000–400 cm^{-1}. These bands are caused by specific vibrations of functional groups and bonds within the protein. These bands can be used to determine protein concentration. During protein hydrolysis, the intensity of the mentioned bands changes, that allows to determine the degree of hydrolysis.

Measurement: 5 µl of sample was mounted on the diamond ATR crystal and a thin dry film was obtained by slow evaporation of the solvent under ambient conditions. The 64 scans, covering a wavenumber range of 4000–400 cm^{-1}, were collected using a spectral resolution of 2 cm^{-1}. The measurements were performed at 20–22 ºC temperature, immediately after drying the sample (approximately 10 min). The same sample was measured 3 times (including separate sample loading on diamond crystal). Raw IR spectra were pre-processed by baseline correction and atmospheric compensation. The intensity of three amide regions was integrated separately (*Amide A* area in the range 3360-3230 cm^{-1}, *Amide I* – 1700-1600 cm^{-1}, *Amide II* – 1600-1480 cm^{-1}) and protein concentration was calculated according to equations 1–3.

Calibration of the method: The method calibration was done using bovine serum albumin (BSA) (2.2.5) as a protein standard. According to each of three amide bands, the calibration curves and equations for measurement of protein concentration were separately established (Supplement 1). To evaluate reliance and repeatability, each calibration point was measured five times.

Calculation:

Amide A $C = \frac{Area}{1.3509} \times d_f$ (1)

Amide I $C = \frac{Area}{1.6162} \times d_f$ (2)

Amide II $C = \frac{Area}{1.5456} \times d_f$ (3)

C – protein concentration (mg/ml);
Area – the integrated area of appropriate Amide region obtained from ATR-FTIR spectrum;
$\mathbf{d_f}$ – dilution factor.
The coefficients were calculated from the calibration curves using BSA.

2.3.2 Estimation of the degree of hydrolysis (DH)

The degree of hydrolysis was estimated according to the measured protein concentrations based on *Amide I* region (2). The DH is described as a ratio of protein concentration before and after hydrolysis (4).

$$DH = \frac{C_1 \times 100\%}{C_2} \quad (4)$$

DH – degree of hydrolysis, %
$\mathbf{C_1}$ – initial protein concentration before hydrolysis reaction, mg/ml;
$\mathbf{C_2}$ – protein concentration after appropriate time of hydrolysis, mg/ml

2.3.3 Determination of protease activity using N,N-dimethylcasein

N,N-dimethylated casein (DMC) is used as a substrate to measure protease activity. During the hydrolysis reaction, the protease hydrolyzes peptide bonds of protein. As a result, peptides and amino acids are released. To quantify the liberated primary amino groups, the TNBS reagent is used. This reagent reacts specifically with the primary amino groups and forms colored complexes, which are have maximum light absorption at 425 nm wavelength. Protease activity is determined by measuring the light intensity at 425 nm.

Measurement: The reaction was performed in a 10 ml conical tube, where 1 ml of enzyme solution and 1.5 ml of BB9 buffer (2.2.6) were added. The solution was thoroughly mixed and pre-warmed for 2 min at 50°C in a thermostatic water bath. Subsequently, 1 ml of substrate solution (DMC) (2.2.6) and 0.25 ml of TNBS (2.2.6) solution were simultaneously added, and the reaction was carried out for 25 min at 50 °C. After the specified time, the tube was removed from the thermostatic water bath, and the reaction was stopped by adding 2.5 ml of ice-cold distilled H_2O. After 20 min of incubation at 20 – 22 °C, the optical density of the sample was measured at $\lambda = 425$ nm.

Control: A parallel control reaction was carried out under identical conditions as the test samples, with the exception that 1 ml of BB9 buffer (2.2.6) was added to the test tube instead of the substrate. The enzyme activity (U/ml) is determined using the (5) formula.

$$Activity = \frac{Abs}{tg\ \alpha} \times \frac{V_R}{V_E} \times \frac{1}{25} \quad (5)$$

Abs –absorption at 425 nm;
tg α – slope of the calibration curve;
V_R – Total reaction volume, ml;
V_E – Enzyme volume, ml;
25 – Duration of reaction, min;

One enzymatic activity unit (1 U) is defined as the amount of enzyme that catalyses the hydrolysis reaction of 1 µmol of N,N-dimethylated casein per minute at pH 8.0 50 ˚C.

Calibration: The calibration curve of primary amino groups was done using glycine solution (2.2.6) as a standard. The calibration curve is provided in supplement 2.

2.3.4 Setting the parameters of alfalfa hydrolysis reaction conditions using commercial enzymes

0,15 g of alfalfa meal was suspended in 5 ml of 0.01 M appropriate pH phosphate buffer (K_2HPO_4) (2.2.7). The obtained solution was centrifuged for 30 s at 3000×g and supernatant was transferred to 15 ml vial. Enzymes solution (*Vilzim Pro AD3000*, *Vilzim Pro NL200*, *Vilzim Pro AL650*) was added to the vial at appropriate quantities. The reaction was performed at a range of temperatures and duration of the hydrolysis listed below, shaking at 200 rpm.

The reactions were performed under these parameters:

- pH – 7.0, 7.5, 8.0, 9.0, 10.0
- Temperature – 25 °C, 35 °C, 50 °C
- Duration of hydrolysis: from 3 to 10 h
- Amount of enzyme (w/w) – 5%, 7%, 10%, 15%, 20%

Controls:

- Control of alfalfa meal – 0,15 g of alfalfa meal was suspended in 5 ml of 0.01 M appropriate pH phosphate buffer (K_2HPO_4) (2.2.7).
- Control of enzyme – an appropriate amount of enzyme was added into 5 ml of 0.01 M appropriate pH phosphate buffer (K_2HPO_4) (2.2.7).

2.3.5 Assessment of sodium nitrite influence to microbial growth during hydrolysis of alfalfa meal

0,15 g of alfalfa meal was suspended in 5 ml of 0.01 M pH 7.0 phosphate buffer (K_2HPO_4) (2.2.7). The obtained solution was centrifuged for 30 s at 3000×g and supernatant was transferred to 15 ml vial. 10% (v/v) of *Vilzim Pro AD3000* was added to the vial. Additionally, sodium nitrite (2.2.7) was supplemented to the final concentration of 1000 µg/ml. The reaction was performed at pH 7.0 35 °C for 6 h shaking at 200 rpm. A parallel control reaction was carried out under identical conditions as

the test samples, omiting sodium nitrite. 50 µl of both samples were plated on LB medium (2.2.8) at different hydrolysis intervals: at initial reaction time, after 3 h and after 6 h of hydrolysis. The plates with the samples were incubated at 37 °C for 48 h.

2.3.6 Polymerase chain reaction (PCR)

The amplification of DNA fragments was performed using PCR. The reaction conditions were selected according to the recommendations of polymerase's manufacturer. The reactions were performed in a thermocycler. The program of PCR was set according to the length of the fragment (Table 2.4). The reaction composition is present in Table 2.4. Total reaction volume – 50 µL.

Table 2.3 The program for PCR using Phusion polymerase.

Cycle step	Temperature	Time	Cycles
Initial denaturation	98 °C	30 s	1
Denaturation	98 °C	10 s	30
Annealing	**X**	30 s	
Extension	72 °C	**Y**	
Final extension	72 °C	5 min	1
Hold	20 °C	∞	∞

X – primer annealing temperature was calculated using manufacturer's calculator[5]
Y – time of extention depends on the speed of the polymerase (Phusion – 15-30 s/kb) and the length of the amplified sequence

Table 2.4 The composition of the PCR reaction mixture.

Reagent	Volume, µL
MiliQ H_2O	19.5
Phusion Hot Start II High-Fidelity PCR Master Mix (2×) (2.2.4)	25
Forward primer	2.5
Reverse primer	2.5
DNA*	0.5

*Optimal DNA concentration is 1 pg-10 ng per reaction volume 50 µL

2.3.7 Preparation of competent *E. coli* cells

E.coli DH5α strain was inoculated in 5 ml of LB medium (2.2.8) and incubated at 37 °C for 16-18 h shaking at 220 rpm. 50 µl of overnight culture was inoculated to 5 ml of fresh LB medium (2.2.8) and incubated at 37 °C for 2 h shaking at 200 rpm until the optical density (OD) at 600 nm in the medium reached 0.4-0.5. Once the OD was appropriate, the cells were chilled on ice for 10 min. Then, the cells were collected into several tubes and centrifuged for 30 s at 6000×g and 4 °C. The supernatant was carefully removed, and the cells were resuspended with 1 ml of ice-cold NaCl solution (2.2.9). Then solution was centrifuged for 30 s at 6000×g and 4 °C and supernatant was discharged. The pellets were further resuspended in 1 ml of ice-cold $CaCl_2$ solution (2.2.9) and centrifuged for 30 s at 6000×g and 4 °C. This step was repeated twice. After that, the supernatant was

[5] https://www.thermofisher.com/lt/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/tm-calculator.html

discarded, and the cells were suspended in 1 ml of ice-cold $CaCl_2$ solution (2.2.9) and were incubated for 1 h on ice. After incubation, the cells suspension was centrifuged for 30 s at 6000×g 4 °C, the supernatant was discarded, and the pellet was resuspended in 50 µL of $CaCl_2$ solution (2.2.9).

2.3.8 Transformation of *E.coli* cells

2-6 µL of DNA was added into competent *E.coli* cells solution and the mixture was incubated on ice for 1 h. After the incubation, heat shock was performed at 42 °C for 45 s in water bath. After the heat shock, *E.coli* cells were returned on ice and incubated for 2 min. After incubation, 1 ml of fresh LB medium (2.2.8) was poured on the cells for recovery and were further incubated at 37 °C for 50 min shaking at 220 rpm. Afterwards, the cells were centrifuged, the supernatant was discarded and *E.coli* cells were spread on the LB agar plates (2.2.8) containing 100 µg/ml of ampicillin (2.2.8). The transformants were grown at 37 °C for 16 h.

2.3.9 Colony PCR

Colony PCR was used for screening transformants. It determines the presence or absence of the transformed DNA. Individual transformants were lysed in water with a short heating step. Then each colony sample was added into the reaction mixture which consisted of the components presented in the table below (Table 2.5). Total reaction volume – 15 µL. The colony PCR was performed using PCR DreamTaq Green PCR Master Mix (2×). The program used for PCR was set according to the length of the fragment (Table 2.6).

Table 2.5 Composition of the PCR reaction mixture including *Taq* polymerase

Reagent	Volume, µL
MiliQ H_2O	5.5
DreamTaq Green PCR Master Mix (2×) (2.2.4)	7.5
Forward primer	0.75
Reverse primer	0.75
DNA*	0.5

*DNA of the colonies from the plate

Table 2.6 The program for PCR using *Taq* polymerase

Cycle step	Temperature	Time	Cycles
Initial denaturation	95 °C	1 min	1
Denaturation	95 °C	30 s	30
Annealing	**X**	30 s	
Extension	72 °C	**Y**	
Final extension	72 °C	5 min	1
Hold	20 °C	∞	∞

X – primer annealing temperature was calculated using manufacturer's calculator
Y – sequence extension time depends on the speed of the polymerase (*Taq* polymerase – 1 min/kb) and the length of the amplified sequence

2.3.10 Purification of plasmid DNA from bacterial cells

The plasmid DNA purification from bacteria was carried out using the commercial kit "GeneJET Plasmid Miniprep Kit" (2.2.4). The bacterial culture was harvested by centrifugation at 6800 × g for

2 min at room temperature. The supernatant was decanted, and the cells from 5-10 ml of culture were resuspended in 250 µl of "Resuspension Solution" by pipetting. Next, 250 µL of "Lysis Solution" was added, and the suspension was thoroughly mixed by inverting the tube 4-6 times until the solution became slightly clear. To prevent the denaturation of supercoiled plasmid DNA, the incubation time should not exceed 5 min. To the suspension, 350 µL of "Neutralization Solution" was added and immediately mixed by inverting the tube 4-6 times. The mixture was then centrifuged for 5 min at 12000×g, and the supernatant was transferred to the "GeneJET" spin column (important to avoid the disturbance of white precipitate). The "GeneJET" column was centrifuged for 1 min at 12000×g, and the flow-through was discarded. Next, 500 µL of "Wash Solution" was added to the "GeneJET" column, and it was centrifuged for an additional 1 min at 12000×g. The flow-through was discarded, and the wash step was repeated using an additional 500 µL of "Wash Solution". The remaining residual "Wash Solution" was collected by performing an additional column centrifugation for 1 min at 12000×g. Then, the "GeneJET" column was transferred into a fresh microcentrifuge tube, and 50 µl of the prewarmed (70 °C) "Elution Buffer" was added to the center of the spin column membrane to elute the DNA plasmid. After incubating for 2 min, the column with the collection tube was centrifuged for 2 min at 12000×g. The extracted plasmid solution was stored at -20 °C.

2.3.11 Restriction of DNA

Restriction was performed using restriction endonucleases (2.2.4). The 20 µl of restriction mixture was consisted of listed components: 2 µl of 10×buffer for appropriate endonuclease, 1 µl of restriction endonuclease (10 U/µL), 0.5-2 µl of DNA (up to 1 µg), MiliQ H_2O to the final reaction volume up to 20 µL. The conditions of the reaction were selected according to protocol provided by manufacturers.

2.3.12 Preparation of electrocompetent *Pichia pastoris* cells and electroporation

Pichia pastoris was inoculated in 20 ml of YEPD medium (2.2.10) and incubated at 30 °C for 18 h shaking at 200 rpm. 4 ml of overnight culture was inoculated to 20 ml of fresh YEPD medium (2.2.10) and incubated at 30 °C for 3 h shaking at 200 rpm until the optical density (OD) at 600 nm in the medium was reached 0.7-0.8. The obtained cells were centrifuged at 3000×g for 5 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 4 ml of YEPD/HEPES solution (2.2.11), supplemented with 0.1 ml of DTT (2.2.11). The resuspended cells were incubated at 30 °C for 15 min without shaking. After incubation, 15 ml of ice-cold sorbitol was added to the mixture and the step of centrifugation was repeated. The washing step using ice-cold sorbitol (2.2.11) was done twice and the finally the cells were suspended in 200 µl of ice-cold sorbitol (2.2.11).

Electroporation. The prepared electrocompetent cells were incubated on ice together with the electroporation cuvettes for 10 min. Up to 0.1 µg DNR samples to be electroporated was added in 40 µl of competent cells and the mixture was transferred to a cold cuvette (it is important to assure that

all cells reach the bottom of cuvette). Afterwards, the cuvette was placed in an electroporator for a high-voltage electric shock (2000 V/0.2 cm, 25 ms, exponential decay). After electroporation, the cells were immediately resuspended in 1 ml of ice-cold sorbitol (2.2.11). In the case when auxotrophic mutants were screened, the cells were plated immediately onto minima agar plates (2.2.11) lacking the appropriate nutrient. In the case when antibiotic resistant transformants were screened, the electroporated cells were incubated at 30 °C for 1h and then were plated on YEPD agar (2.2.10) with appropriate antibiotic. In both cases, the cells were incubated at 30 °C for 72 h.

2.3.13 Protein concentration

Amicon® Ultra 30K centrifugal filter concentrator was used for protein concentration. The concentration was performed according to recommendations provided by manufacturers (Milipore").

2.3.14 Cell disruption by glass beads method

The pre-weighed cells were resuspended in an appropriate volume of BB9 pH 9.0 buffer (2.2.6) (0.1 g of cells: 700 µL of buffer). Then, 100 mM PMSF was added to the cell suspension to a final concentration of 1 mM. All subsequent procedures were conducted on ice. Next, 0.5 g of glass beads were added to the cell suspension, and the cells were disrupted by vortexing for 30 s followed by incubation on ice for 30 s. This disruption cycle was repeated for a total duration of 7 min. After disruption, the supernatant was carefully transferred to a clean 1.5 ml tube and centrifuged at 17,000×g for 10 min at 4 °C. After centrifugation, the soluble protein fraction (supernatant) was separated from the insoluble protein fraction (pellet). The insoluble protein fraction was then resuspended in 1 ml of BB9 pH 9.0 buffer (2.2.6) and centrifuged again at 17,000×g for 10 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in the same volume of BB9 pH 9.0 buffer (2.2.6) as the soluble protein fraction volume.

2.3.15 SDS-PAGE electrophoresis

The inner surfaces of the glass plates were cleaned with 99.8% ethanol prior to the assembly of the electrophoresis frame. Then, a 12% separating gel was prepared according to Table 2.7. TEMED and APS (2.2.12) were added as the last components to the mixture. The obtained gel solution was quickly poured into the assembled electrophoresis frame. Distilled H_2O was poured on top, and the gel was left to solidify for 30 min. Afterward, the water was removed, and a 4% stacking gel was poured on top of the separating gel. Combs of the desired size were carefully placed, and the gel was allowed to polymerize for 30 min. After polymerization, the combs were removed, and the electrophoresis frame with the gel was transferred to a vertical protein electrophoresis chamber filled with 1x electrophoresis buffer solution (2.2.12). The electrophoresis was conducted at a voltage of 150 V.

The samples for electrophoresis were prepared by mixing the protein sample with 2x electrophoretic dye (2.2.12) at a ratio of 1:1. Then, the mixture was heated for 5 min at 95 °C. 2-20 µL of the sample was loaded into the wells, along with 3 µL of protein molecular weight marker (2.2.4). After electrophoresis, the gel was stained with "Commassie Blue R-250" dye.

Table 2.7 The composition of stacking gel and separating gel used for SDS-PAGE electrophoresis.

12 % separating gel	4 % stacking gel
2000 µL 30% AA ir BAA solution	335 µL 30% AA ir BAA solution
1250 µL 1,5 M Tris-HCl (pH 8,8)	625 µL 1,5 M Tris-HCl (pH 6,8)
50 µL 10% SDS	50 µL 10% NDS
1585 µL dH_2O	1500 µL dH_2O
25 µL 10% APS	12,5 µL 10% APS
2,5 µL TEMED	2,5 µL TEMED

2.3.16 DNA electrophoresis

DNA gel electrophoresis was performed using 1% agarose. 1 g of agarose was dissolved in 100 ml of 1×TAE buffer (2.2.13) and heated in a microwave until the agarose dissolved. The solution was left to cool down for approximately 5 min, reaching a temperature of about 50 °C. Then, 5 µl of ethidium bromide (2.2.13) was added, and the solution was poured into a gel casting tray with the well comb in place and left to solidify for 20 min. The obtained agarose gel was placed in a horizontal electrophoresis box, and the box was filled with 1×TAE buffer (2.2.13). The samples were prepared by mixing with 6× loading dye (2.2.3) in a ratio of 5:1. The prepared samples were loaded into the wells of the agarose gel. For DNA size determination, 3 µL of molecular weight marker were loaded into one of the gel's wells. Electrophoresis was performed for approximately 30 min at a set voltage of 7 V/cm. After electrophoresis, the results were analyzed using a UV transilluminator.

Preparative DNA electrophoresis was performed for the extraction of the DNA fragments from agarose gel. The gel was prepared in a horizontal electrophoresis chamber and once the gel solidified, the camber was filled with fresh 1×TAE buffer (2.2.13). The voltage used for preparative electrophoresis was 3-5 V/cm.

2.3.17 Purification of DNA fragments after PCR or enzymatic restriction

The "GeneJET PCR Purification Kit" (2.2.4) was utilized for purifying DNA after PCR or DNA restriction. The completed PCR mixture was thoroughly mixed with "Binding Buffer" in a 1:1 (w/w) ratio. If the DNA fragment size was ≤500 bp, 1:2 volume of 100% isopropanol was added. The resulting solution was transferred to the "GeneJET" purification column and centrifuged for 1 min at 12000×g. The flow-through was discarded. Next, 700 µl of Wash Buffer was added to the column,

and it was centrifuged for 1 min at 12000×g. The wash procedure was repeated one more time. The flow-through was discarded, and any remaining residual "Wash Solution" was collected by performing an additional column centrifugation for 1 min at 12000×g. Afterwards, the "GeneJET" purification column was placed in a clean 1.5 ml microcentrifuge tube, and 50 µl of prewarmed (65°C) "Elution Buffer" was added to the center of the GeneJET purification column membrane. Finally, the column was centrifuged for 1 min at 12000×g, and the purified DNA solution was stored at -20 °C.

2.3.18 Extraction of DNA from agarose gel

The "GeneJET Gel Extraction Kit" (2.2.4) was used for DNA extraction from an agarose gel. After preparative electrophoresis, the DNA fragment was excised from the gel. The gel slice was placed in a pre-weighed 1.5 ml tube and its weight was recorded. The excised gel slice was mixed with "Binding Buffer" in a 1:1 volume-to-weight ratio. The mixture with the gel was incubated at 60 °C for 10 min until the gel slice completely dissolved. Once the gel was solubilized, the obtained solution was transferred to a "GeneJET" purification column and centrifuged for 1 min at 12000×g. The flow-through was discarded. Next, 700 µL of "Wash Buffer" was added to the column and centrifuged for 1 min at 12000×g. The flow-through was discarded, and centrifugation was repeated for an additional 1 min at 12000×g to remove the residuals of the "Wash Buffer". After that, the "GeneJET" purification column was placed into a clean 1.5 ml microcentrifuge tube and 50 µl of prewarmed (65°C) "Elution Buffer" was added to the center of the “GeneJET” purification column membrane. The tube with the column was incubated for 1 min and then centrifuged for 1 min at 12000×g. The purified DNA solution was stored at -20 °C.

2.3.19 Measurement of DNA concentration

DNA concentration was measured using spectrophotometer „Nanodrop” (Thermo Fisher Scientific) according to the manufacturer’s instructions.

2.3.20 Prolonged overlap extension PCR

Prolonged overlap extension PCR (POE-PCR) was employed for multiple site-directed mutagenesis. The primers were designed to have overlapping sequences with each amplified fragment, and each primer also included a mutation flanked by the overlap region. Initially, conventional PCR (2.3.6) was conducted to amplify individual DNA fragments using the designed primers (2.2.2). The resulting fragments with overlapping regions were purified using the GeneJET PCR Purification Kit (2.3.17). The POE-PCR reaction mixture consisted of equimolar amounts of each fragment (4nM), and 12.5 µl of Phusion Hot Start II High-Fidelity PCR Master Mix (2×) (2.2.4) was used for the reaction. The specific program utilized for the POE-PCR is presented in Table 2.8.

Table 2.8 The program used for POE-PCR using Phusion polymerase

Cycle step	Temperature	Time	Cycles
Initial denaturation	98 °C	30 s	1
Denaturation	98 °C	10 s	30
Annealing	60 °C	15 s	
Extension	72 °C	2min 42 s	
Final extension	72 °C	10 min	1
Hold	20 °C	∞	∞

2.3.21 Golden Gate assembly (GGA)

The Golden Gate assembly was performed according to scientific literature (Prielhofer et al., 2017; Marillonnet & Grützner, 2020). The composition of the reactions is provided in Supplement 3. The GGA reaction was performed in thermocycler using the program listed below:

Cycle step	Temperature	Time	Cycles
Digestion	37 °C	2 min	30
Ligation	16 °C	2 min	
Final digestion	37 °C	10 min	1
Final extension	55 °C	30 min	
Heat inactivation	80 °C	10 min	

3 RESULTS AND DISCUSSION

The goal of the study was to develop a biocatalytic technology for the hydrolysis of plant-derived proteins. The investigation consisted of two parts: i) setting the parameters for the hydrolysis process catalysed by commercial enzymes, and ii) the development of recombinant protease.

Alfalfa (*Medicago sativa* L.), a perennial herb belonging to the legume family, was chosen as a plant of interest. Alfalfa meal was used as a source of proteins for the hydrolysis. It is one of the most used feed for livestock because of its superior content of vitamins, minerals, and proteins compared to other feed sources. In recent years it started to be used more often in human nutrition as an inexpensive source of proteins for supplements or replacement of expensive animal proteins (Apostol et al., 2017). Alfalfa contains many nutrients, including minerals (calcium, phosphorus, iron, magnesium, potassium, zinc, copper, selenium, organic silicon, manganese), vitamins (C, K, D, E, U, provitamin A, B1, B2, B6, B12, folic acid/B9, biotin, niacin), as well as β-carotene and essential amino acids. Alfalfa is a rich source of proteins; in a dry matter it contains 15% of proteins in stems and 35% in leaves. Good balance of its essential amino acids composition corresponds to FAO recommendations for daily human value of protein consumption. Protein hydrolysates are the most nutritious forms of proteins, therefore, the alfalfa plant is the potential sources for protein hydrolysate production.

3.1 Development of ATR-FTIR spectroscopy-based method for assessment of hydrolysis process

First of all, the method based on ATR-FTIR spectroscopy was developed to assess the hydrolysis process. ATR-FTIR spectroscopy is a label-free technique that can be tailored to various biological applications. During measurements, infrared radiation, reflected by a crystal with a high refractive index, is partially absorbed by the sample and finally collected by a detector (Ferro et al., 2019). Absorbed wavelengths depend on the molecular vibrations within the substance. The sample's infrared absorbance can determine both chemical and structural information from the sample material. ATR-FTIR spectroscopy method is quick and non-destructive method, it requires minimal sample preparation, and also it could be used for analysis of complex and heterogeneous samples and solutions (Tiernan et al., 2021). There are a number of reports where ATR-FTIR technique is used for imaging of protein secondary structure, however, there is a lack of information about ATR-FTIR application for monitoring protein hydrolysis process.

The infrared spectrum of protein exhibits three major characteristic bands which are caused by specific vibrations of functional groups and bonds within the protein (Fig 3.1).

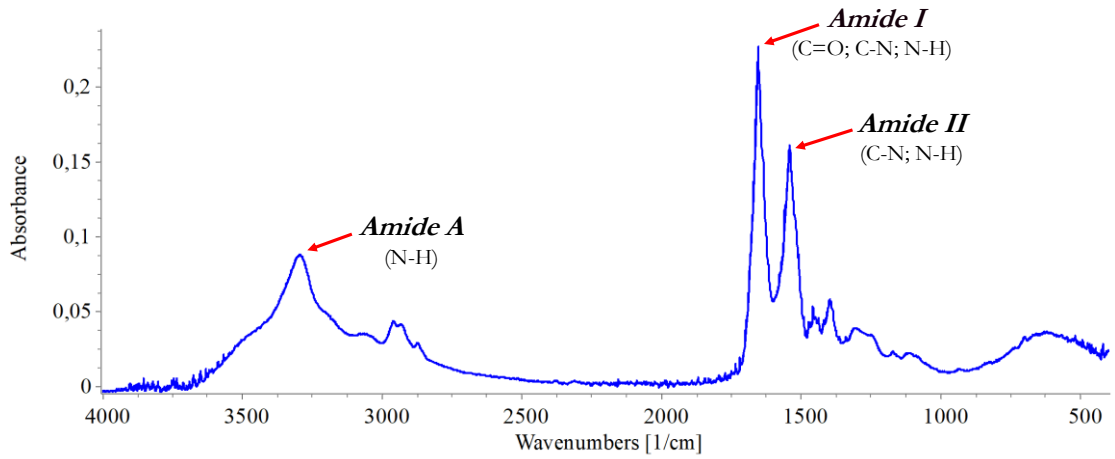


Fig. 3.1 The ATR-FTIR spectrum of BSA protein.

They are known as *Amide* bands, which are most used in protein structure analysis. The *Amide A* band occurs at 3310-3270 cm^{-1} and is mainly derived from N-H bond vibration (Table 3.1).

Table 3.1 The characteristics of *Amide A*, *Amide I* and *Amide II* bands and their equations for estimation of protein concentration. c – protein concentration (mg/ml); Area – the integrated area obtained from ATR-FTIR spectrum; d_f – dilution factor.

Band	Wavenumbers, cm^{-1}	Vibrations	Sensitivity to structural changes	Equation
Amide A	3310-3270	N-H	The least sensitive	$c(mg/ml) = \frac{Area}{1.3509} \times d_f$
Amide I	1600-1700	C=O; C-N; N-H	The most sensitive	$c(mg/ml) = \frac{Area}{1.6162} \times d_f$
Amide II	1500-1600	C-N; N-H	Less sensitive	$c(mg/ml) = \frac{Area}{1.5456} \times d_f$

The *Amide I* band, which has the strongest absorption of infrared light, can be observed between 1600-1700 cm^{-1}. It is caused by stretching vibrations of C=O coupled with C-N stretch and N-H bending. The *Amide II* band is mainly caused by C-N stretch along with N-H in-plane bending and occurs at 1500-1600 cm^{-1}. The study by Barth & Zscherp has indicated that simple and known structure homopolypeptides had exposed relationship between *Amide I* band position and secondary structure of peptide (Barth & Zscherp, 2002). All three bands could be used to study the structure of proteins; however, the *Amide I* band is the most commonly used in the analysis of secondary structure due to its sensitivity to changes in secondary structure.

After calibration with bovine serum albumin (BSA) as a protein standard, the protein concentration of hydrolysates was investigated by ATR-FTIR spectroscopy (2.3.1). The equations for estimation of protein concentration were established separately for each *Amide* band. One of the challenges of this method is the inability to measure the aqueous samples of proteins, because the spectra of the water overlap with the *Amide I* band at 1600 cm^{-1}. To overcome this issue, the air-dried preparation of samples was applied for aqueous protein samples.

During the hydrolysis reaction, proteases hydrolyse peptide bonds between amino acids. Cleavage of peptide bonds causes changes in protein structure, which are notable in the ATR-FTIR spectra. For the validation of the ATR-FTIR method, skimmed milk powder (2.2.3) was used as a substrate for hydrolysis reaction. Prior to the hydrolysis, protein content in skimmed milk was evaluated. The prepared 9.52 mg/ml skimmed milk solution was measured by ATR-FTIR method (2.3.1). The estimated *Amide I* and *Amide II* concentrations were similar to theoretical value - 11.03 ± 2.04 mg/ml and 10.54 ± 1.90 mg/ml, respectively. The concentration of *Amide A* band was significantly higher - 25.55 ± 1.4 mg/ml. This discrepancy might occur due to non-protein origin components found in skimmed milk mixture, that gave additional signals and vibrations in the same range.

Further, skimmed milk hydrolysis was performed using commercial protease *Vilzim PRO AD3000* for 4 h at 20˚C. The ATR-FTIR spectra were measured after each hour of hydrolysis and the degree of hydrolysis (DH) to each *Amide* band was estimated (2.3.2) (Fig 3. 2).

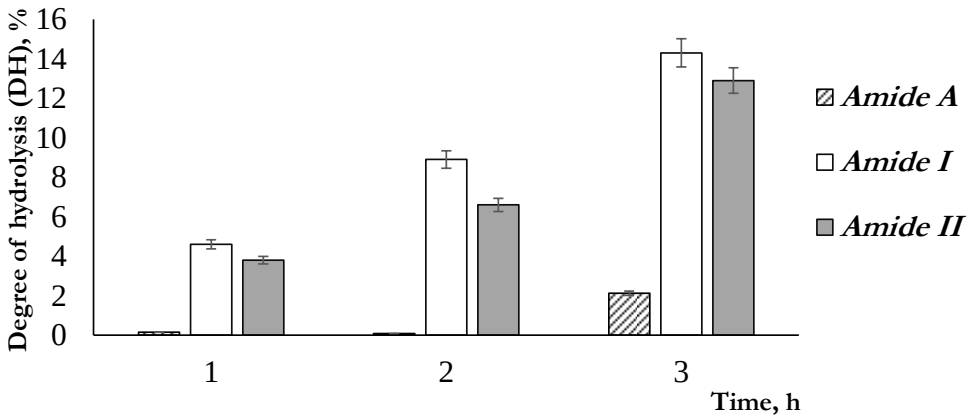


Fig 3.2 The hydrolysis of skimmed milk catalysed by commercial protease *Vilzim PRO AD3000.*

During the hydrolysis, the DH of *Amide I* and *II* has increased over time. After 3 h of hydrolysis, the DH of *Amide I* and *II* has reached 14.3% and 12.9%, respectively; whereas the DH of *Amide A* has reached only 2%. The results confirmed the fact that *Amide A* is the least sensitive to protein backbone conformation, because the N-H vibrations is mainly dependent on the strength of a hydrogen bond (Krimm & Bandekar, 1986). Oppositely, the decreased bands of *Amide I* and *II* proved that the method is reliable and suitable for monitoring the cleavage of peptide bonds during the hydrolysis reaction. Since the band of *Amide II* is less sensitive to structural changes, for further experiments, the results of *Amide I* was chosen to assess in this study, although, all bands were followed and analysed.

3.2 The hydrolysis of alfalfa meal by commercial enzymes

The assessment of alfalfa meal hydrolysis process was started with the selection of the most suitable commercial proteases. Three different proteases were tested. To evaluate the catalytic properties of proteases, enzymatic activity was estimated of these proteases (2.3.3). General

information provided by the manufacturer and measured catalytic activities of the enzymes are listed in Table 3.2.

Table 3.2 Characteristics of commercial proteases.

	Vilzim PRO AD3000	***Vilzim PRO AL650***	***Vilzim PRO NL200***
Type of protease	Alkaline	Alkaline	Neutral
Form	Powder	Liquid	Liquid
pH stability	6.0 – 10.0	6.0 – 10.0	5.0 – 10.5
Temperature stability	60 – 70 ˚C	60 – 70 ˚C	60 – 70 ˚C
Estimated proteolytic activity*	1557 U/ml	253 U/ml	197058 U/ml

*Relative standard deviation not exceed < 5%.

Neutral protease *Vilzim Pro NL200* had the highest proteolytic activity – 197058 U/ml. The estimated alkaline protease *Vilzim Pro AD3000* activity was 1557 U/ml, and the lowest activity was observed of alkaline protease – *Vilzim Pro AL650* – 253 U/ml. Further, three hydrolysis reactions were performed using the above mentioned proteases at 25 ˚C (2.3.4). The degree of hydrolysis was followed for 3 h to evaluate the reaction efficiency. The obtained results are presented in Fig 3.3.

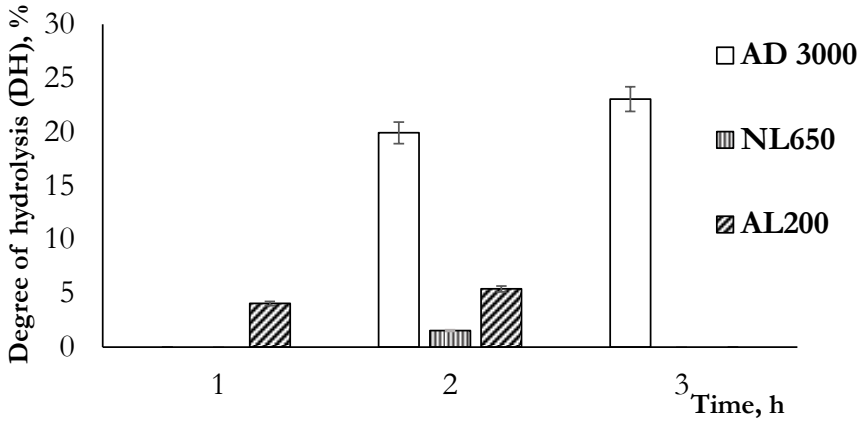


Fig 3.3 The hydrolysis of alfalfa meal using three different commercial proteases.

After 3 h, only one of the three hydrolysis reactions gave significant results. The highest DH of 23% was determined when *Vilzim Pro AD3000* was employed for the hydrolysis. The other two proteases, *Vilzim Pro NL200* and *Vilzim Pro AL650,* performed the hydrolysis less intensively, the defined DH was 5% and 2%, respectively. It is worth to mention, that the latter both proteases were in a liquid form. Usually, commercial enzymes contain various additives. Some of them are hygroscopic compounds that can hinder the evaporation process. During the ATR-FTIR measurement, the drop of the sample could not evaporate completely, and the spectrum of the protein sample may overlap with the water region that covers the required bands of protein spectrum. This could be one the possible explanations for the obtained significantly low DH in the reactions with *Vilzim Pro NL200* and *Vilzim Pro AL650*. For further investigation of alfalfa hydrolysis, the protease *Vilzim Pro AD3000* was chosen.

3.3 Setting of process parameters for biocatalytic alfalfa hydrolysis

Each enzyme has a certain range in temperature and pH in which a maximal rate of reaction is achieved. It is called the optimum of a temperature or a pH. They have a significant influence on the activity of enzymes and the impact for the whole hydrolysis process (Merz et al., 2015). The first parameter assessed for hydrolysis process was pH. The buffered systems were used for keeping the pH stable during the whole hydrolysis reactions. According to the manufacturer's specification, *Vilzim Pro AD3000* has a wide range of pH stability 6.0 – 10.0, however the optimal pH was not provided. For estimation of the optimal pH of the process hydrolysis reactions were performed at different pH values: 7.0, 7.5, 8.0, 9.0, and 10.0 (2.3.4) (Fig 3.4).

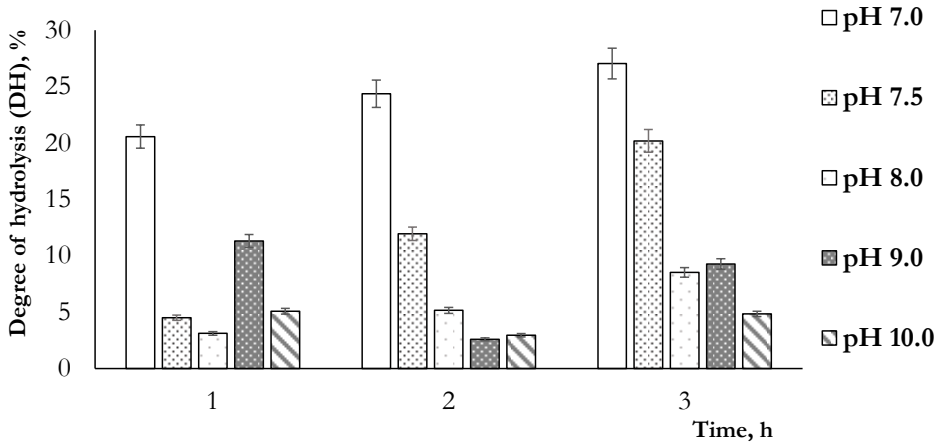


Fig 3.4 The influence of pH for the enzymatic hydrolysis of alfalfa meal.

The highest DH of 27% was obtained when the reaction was performed at pH 7.0. The reaction performed at pH 7.5 exposed the lower DH of 20%. The applied higher pH values such as 8.0, 9.0, and 10.0 strongly influenced the hydrolysis process, the determined DH values were lower than 10%. For further assessments of hydrolysis process, it was chosen to perform the hydrolysis reaction at pH 7.0.

The temperature was the second parameter in the hydrolysis assessment list. Each enzyme has an optimal temperature, below or above which the enzyme has low activity or may be even denatured. For enzymatic conversion of substrate into product, enzymes must collide and bind the substrate molecule to their active site. When the temperature increases, the catalytic system speeds up the Brownian motion of the molecules within the reaction solution, thus increasing a chance of collisions between enzymes and substrates per unit of time (Cao & Wang, 2016). Large number of collisions results in a faster reaction, which explains the temperature influence on enzymatic activity. The use of higher than optimal temperatures could result in the disruption of hydrogen bonds that stabilize the structure of the enzyme, thus incorrect refolding, protein aggregation and loss of activity could occur (Fágáin, 1995). The hydrolysis reactions were carried out at three different temperatures: 20 ˚C, 35 ˚C and 50 ˚C (2.3.4) (Fig 3.5).

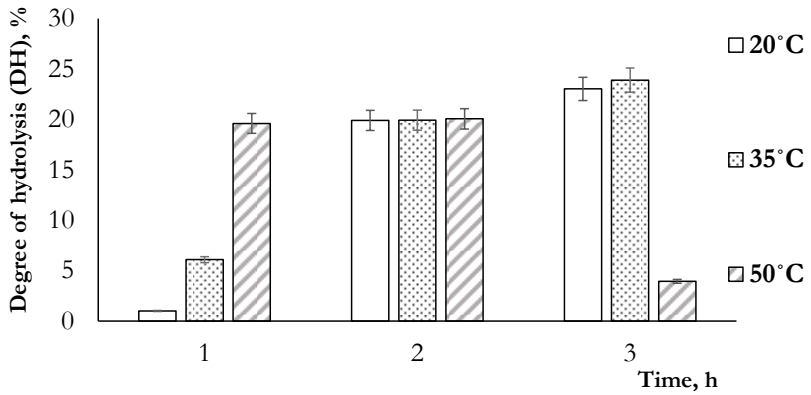


Fig 3.5 The influence of temperature for the enzymatic hydrolysis of alfalfa meal.

After 3 h of hydrolysis, DH was quite similar 23% and 25% at 20 ˚C and 35 ˚C, respectively. At 50 ˚C degrees, the DH after 2 h of incubation was 20%; however, after 3 h it suddenly decreased up to 5%. As proteases are enzymatically active proteins, they are very sensitive to reaction conditions. Thus, for alfalfa hydrolysis, the highest DH was obtained at 35 ˚C, which was applied for further studies.

The amount of catalyst is another important parameter for enzymatically catalysed processes. The reactions were carried out using different amount of enzyme 5%, 7%, 10%, 15% and 20% (w/w) (2.3.4) (Fig 3.6).

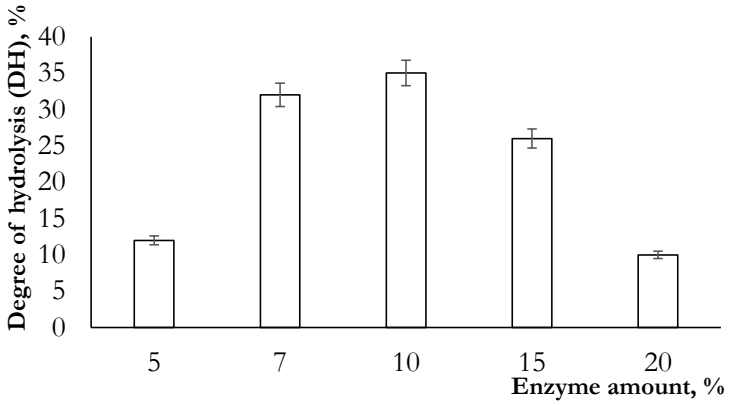


Fig 3.6 The influence of enzyme concentration for the enzymatic hydrolysis of in alfalfa meal.

The highest DH of 35% was determined when the hydrolysis was performed using 10% of enzyme. The other used concentrations resulted in DH lower than expected. Usually, the increase of the amount of catalyst influences the catalytic performance more effectively. However, it was quite unusual to observed that excessive amount of enzymes provides lower DH. Notwithstanding, for further studies of hydrolysis process, 10% of enzyme was used as a constant parameter.

Further, the hydrolysis was performed for 10 h duration and DH was assayed each hour (2.3.4) (Fig 3.7).

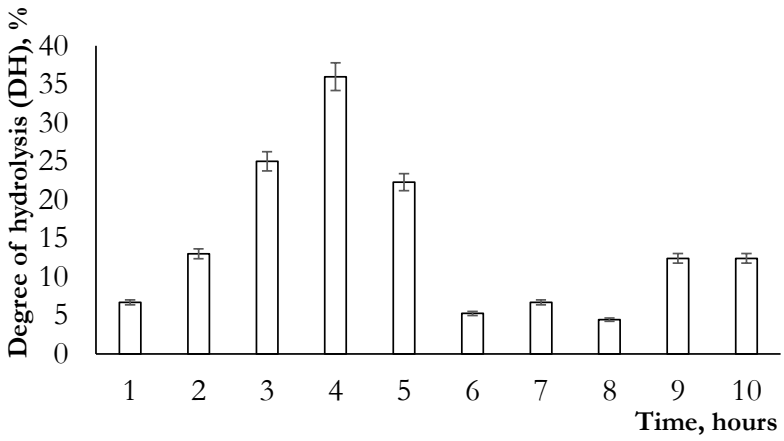


Fig 3.7 The influence of reaction time in alfalfa meal enzymatic hydrolysis process.

The highest DH of 36% was obtained after 4 h of the hydrolysis. Longer hydrolysis time resulted in the decrease of DH. One of the explanations of such decrease of DH could be microbial contamination. The reaction mixture contains various substances such as polysaccharides, fats and proteins that are good nutrients for microorganisms. In addition, the temperature applied for the incubation, is suitable for the growth of microorganisms. Thus, to evaluate the possible microbial contamination, two different media were applied for the experiment – LB (2.2.8) and YEPD (2.2.10). The solutions of alfalfa meal and enzyme powder were assessed. 50 µl of the samples were plated on Petri dishes. The Petri dishes with LB media were incubated at 37 ˚C for 48 h and Petri dishes with YEPD media plates at 30 ˚C for 48 h. It was determined that the main microbiological contamination has come from the alfalfa meal, a lot of colonies were formed on both types of media plates. This experiment revealed that both initial substances, enzyme powder and alfalfa meal, are non-sterile and contain microbial contamination that influence the hydrolysis process in long term experiments.

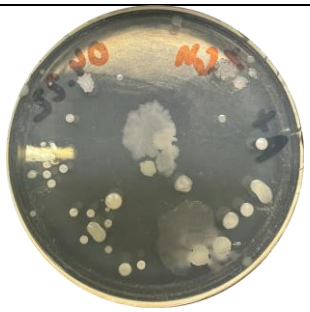
To overcome this issue, several strategies could be applied. Sterilization in autoclave is commonly used to prevent microbial contamination. However, autoclavation is performed under high pressure and temperature, and these conditions can be too harsh for proteins leading to their denaturation (Marciniak et al., 2018). Enzymes cannot be autoclaved, therefore, for their sterilization filtration filters can be applied, for example 0,45 µm PVDF sterile membrane filter.

For sterilization experiments, the alfalfa meal solution was prepared and autoclaved, and enzyme solution was filtered using 0,45 µm PVDF sterile membrane filter. Further, the hydrolysis reaction was performed at the previously defined conditions pH 7.0, 35˚C, 10% of enzyme, hydrolysis duration of 4 h (2.3.4). The results of ATR-FTIR measurement clearly indicated that the concentration of proteins did not change during the hydrolysis. After 4 h of hydrolysis, the DH was 0%. It means that the reaction was not effective. The alfalfa meal, used in the study, contains various substances such as polysaccharides, fibers, fats, etc. During autoclavation, various molecules in the meal mixture might interact with proteins under thermal exposure, thus leading to formation of protein aggregates.

Such aggregates become inaccessible to proteases because of surrounded additives which hinders the accessibility of peptide bonds. Hence, autoclavation is not appropriate sterilization method for proteins of alfalfa meal. To overcome microbial contamination, it was decided to use antimicrobial substances, which could inhibit the growth of microorganisms during the hydrolysis reaction.

In food industry, elimination of microbial contamination by physical methods is not always possible; therefore, antimicrobial compounds (so called agents) are applied. There are a wide variety of compounds that can be used as an antimicrobial agent in food, however, it is important that such compounds have to be approved for use and has zero or low toxicity. Based on the literature, nitrites and nitrates are the compounds that are often used in food for preservation of meat products. To evaluate the inhibition of microbial growth during the hydrolysis reaction, sodium nitrite was chosen as an antimicrobial agent Sodium nitrite concentration of 1000 µg/ml was selected according to the EU regulations for antimicrobial substances used in food. Two hydrolysis reactions were performed in parallel for 6 h: one reaction with sodium nitrite (A+) and another one appointed as a control reaction without the antimicrobial substance (A-) (2.3.5). 50 µl of both samples were placed on LB medium (2.2.8) at different hydrolysis intervals: at initial reaction time, after 3 h and after 6 h. The plates were incubated at 37 ˚C for 48 h. The results are presented in Table 3.3.

Table 3.3 The influence of sodium nitrite (1000 µg/ml) on microbial growth during the hydrolysis of alfalfa meal.

	Duration of hydrolysis		
	0 h	**3 h**	**6 h**
A- ***(control)***			
CFU/ml	2×10^3	9.8×10^2	1.3×10^3
A+			
CFU/ml	1.2×10^3	7.6×10^2	7.8×10^2

At the initial hydrolysis stage, there was a significant difference between A+ plate (2×10^3 CFU/ml) and A- plate (1.2×10^3 CFU/ml). After 3 h of hydrolysis reaction, in both plates, A+ and A-, there was

a diminished number of CFU/ml compared to initial hydrolysis stage – 7.6×10^2 CFU/ml and 9.8×10^2 CFU/ml, respectively. After 6 h of hydrolysis, in A+ sample plate CFU number remained similar as after 3 h of hydrolysis – 7.8×10^2 CFU. On the plate A- the CFU number increased from 9.8×10^2 CFU/ml to 1.3×10^3 CFU/ml.

Two insights could be made of these results. Firstly, comparing microbial growth of the reaction with sodium nitrite and control, lower CFU/ml in A+ plates indicate that antimicrobial substance supresses the growth of microorganisms. However, unusual tendency was observed in control plates where after 3 h of hydrolysis the CFU decreased too, although no antimicrobial substance was added. As it has been mentioned in the literature review, hydrolysed short chain peptides could exhibit various bioactivities, one of them is antimicrobial activity. Some short chain peptides could have been released during the hydrolysis reaction and affect the microbial growth. However, incubation for a longer time results in increased CFU due to low concentration of the peptides with antimicrobial activity. Oppositely, when antimicrobial agents are added into reaction mixture, the microbial growth remains stable. For further studies, higher concentrations or another antimicrobial agent would be recommended to apply.

3.4 Development of recombinant protease

The results of recombinant serratopeptidase (SRP) gene cloning in *E.coli* and its expression in *P.pastoris* will be presented in this section. SRP protease was isolated from *Serratia marcescens* by laboratory colleagues. SRP gene is 1480 bp in length. Two different cloning techniques were applied. The first technique was a traditional cloning method, which relies on the use of restriction endonucleases to generate DNA fragments with specific complementary end sequence. Performing PCR, the gene was amplified, and restriction sites were added to the ends of the sequence. Restriction enzymes were used to cut both, the gene and the vector, and then, DNA ligase was used to compile the ends of both fragments. Pic9K (Invitrogen) vector was used for the cloning. It contains AOX1 promotor that is used for the expression of recombinant protein. Another selected cloning technique was the Golden*Pics* cloning system, which is based on Golden Gate cloning principle. Type IIS restriction enzymes and T4 DNA ligase are used to assemble the expression vector. This method allows to assemble a few expression units on one plasmid with the ability to use various promotors and terminators (Prielhofer et al., 2017).

P.pastoris GS115 strain was chosen as an expression host. It is a methylotrophic yeast, which produces alcohol oxidase enzyme for metabolization of methanol. Alcohol oxidase is coded by two genes – *AOX1* and *AOX2*. The majority of alcohol oxidase activity in *P.pastoris* cells accounts for *AOX1* gene product. Expression of the *AOX1* is firmly regulated and induced by methanol to very

high levels, while *AOX2* gene has weaker induction by methanol. Pic9K vector is one of the *AOX1* promoters containing vector for effective recombinant protein expression.

3.4.1 Conventional cloning method

Heterologous proteins can be expressed in *P.pastoris* as an intracellular protein or can be secreted out into the medium. The main advantage of secretion of heterologous proteins in *P.pastoris* is that this host secretes low amounts of native proteins. Pic9K cloning vector was selected for secreted expression of SRP. The general plasmid parts are present in **Fig 3.8.**

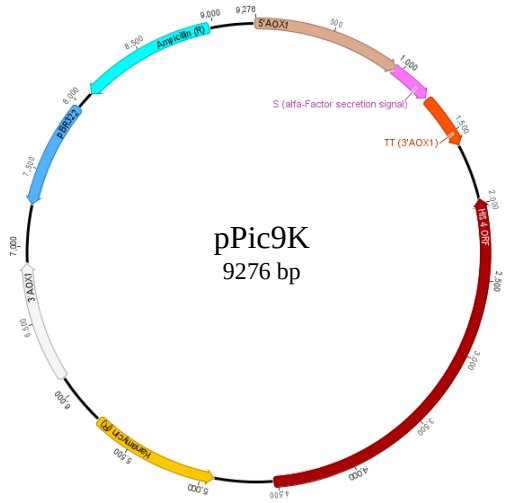


Fig 3.8 The Pic9K plasmid.

Pic9K plasmid has a promotor and terminator of *P.pastoris AOX1* gene, β-lactamase gene which codes protein that is responsible for ampicillin resistance and is used for selection of *E.coli* clones. Also, the vector contains the bacterial kanamycin resistance gene that confers resistance to geneticin (G418) in *P.pastoris*. Since the kanamycin resistance gene is linked to the inserted gene, multiple insertions of kanamycin resistance into *P.pastoris* chromosome increases the resistance to G418, thus, this may also indicate that the recombinant gene is present in multiple copies. Pic9K vector has the α-factor sequence, that subjects the protein into medium, notwithstanding, SRP has its own propeptide sequence, which plays a role of intramolecular chaperons. The propeptide upholds the correct folding of the subsequent catalytic domain and it is cleaved during maturation (Doshi et al., 2022). To assess the native propeptide influence for expression of SRP, it was decided to design two different constructs – with and without a propeptide sequence.

Preparation of SRP gene for cloning into E.coli

The study was started with the construction of expression vector. The primers were designed according to recommendations of the plasmid manufacturer („Thermo Fisher Scientific"). Two forward primers were designed, with and without propeptide sequence of SRP, P708 and P710, respectively (2.2.2). The reverse primers were designed with and without His-tag – P709 and P711, respectively (2.2.2). Addition of the His-tag is the most commonly used approach for latter performance of

heterologous protein purification. However, it might have the influence for the activity of protein, for this reason as control the construct without the His-tags was constituted. Four primers pairs are listed in Table 3.4, which were applied for SRP gene amplification by PCR method (2.3.6).

Table 3.4 Primers pairs used for PCR amplification of SRP gene.

Serratopeptidase constructs	Primers pairs
Construct I – with propeptide, with His-tag	P708/P709
Construct II – with propeptide, without His-tag	P708/P711
Construct III – without propeptide, with His-tag	P710/P709
Construct IV – without propeptide, with His-tag	P710/P711

PCR reactions were performed using *Serratia* sp. genome DNR as a matrix and *Phusion* polymerase („Thermo Fisher Scientific"). All the PCR products were about 1480 bp in length (Fig 3.9).

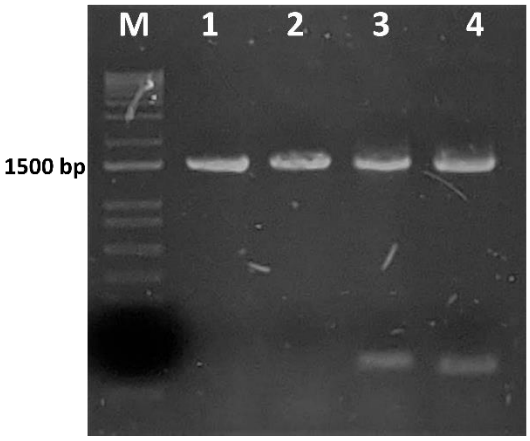


Fig 3.9 PCR products obtained using different primer pairs
M - 1 Kb Plus DNA Ladder **1 lane -** *Construct I*; **2 lane -** *Construct II*; **3 lane -** *Construct III*; **4 lane -** *Construct IV*.

Amplified SRP gene constructs were cloned into Pic9K vector according to the manufacturer's recommendations („Thermo Fisher Scientific"), using *Not*I and *Eco*RI restriction enzymes (2.2.4). The constructed Pic9K-SRP plasmids were used for transformation in a *E.coli* DH5α. Competent cells were prepared (2.3.7) and transformed by chemical transformation (2.3.8). Empty Pic9K plasmid was used as a positive control for transformability; competent cells without any DNA were used as a negative control. Ampicillin was used as selective pressure marker for transformants. Obtained clones were screened by colony PCR method (2.3.9). The primers of AOX1 promotor (P652) and terminator (P653) were used to ensure that the target gene incorporates into the plasmid (2.2.2).

The obtained results of colony PCR have indicated that transformability of clones was successful: 1 of 10 transformed colonies had the plasmid of *construct I*; 3 of 27 transformed colonies had the *construct II* plasmid; 9 of 11 transformed colonies had the *construct III* plasmid; all 11 colonies had the *construct IV* plasmid.

After the colony PCR, the plasmids which contained the constructs were extracted from the *E.coli* DH5α cells and purified using Plasmid extraction kit (2.3.10).

Restriction analysis (2.3.11) of DNA plasmids was done using *Sac*I, *Not*I and *Eco*RI. It confirmed that all plasmids contained the constructs of SRP gene. Further, the plasmids were prepared for sequencing, which was done at DNA Sequence Center, Institute of Biotechnology. Sequencing results revealed that all SRP constructs were correctly inserted into vector and can be used for further experiments.

Serratopeptidase gene transformation into P.pastoris

The insertion of Pic9K expression vector occurs at the histidinol dehydrogenase gene (*his4)* locus in *P.pastoris* genome. The GS115 strain has a mutation in the *his4* that prevents it from the synthesizing of histidine. Expression vector Pic9K has *HIS4* gene that complements the native gene in the host. The integration into genome at the *his4* locus happens from a single cross over occurrence between the *his4* locus in the chromosome and the *HIS4* gene on the vector. Prior to integration, expression vectors have to be linearized. *Sal* I restriction enzyme was used for the restriction (2.3.11) of Pic9K expression vector. Firstly, it was decided to perform transformation of the vector which contains the *construct I.* Linearized plasmid was transformed into *P.pastoris* by electroporation method (2.3.12). The transformability of yeast cells was tested using empty Pic9K vector as a positive control. For negative control, only electroporated cells were applied. Selection of transformants was performed using histidine-deficient medium (2.2.11). The obtained clones on histidine-deficient medium were further screened on Petri dishes with different concentration of geneticin – 0.25 mg/ml, 0.5 mg/ml, 1.0 mg/ml, 1.5 mg/ml and 2.0 mg/ml. Transformants were grown for 3 days at 30 °C. Afterwards, the grown transformants were analysed by colony PCR method (2.3.9), using primers of *AOX1* promotor and terminator – P652 and P653, respectively (2.2.2). Results of colony PCR are presented in Fig. 3.10.

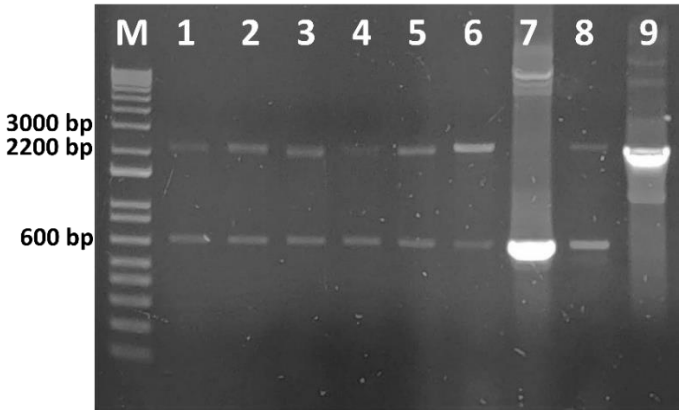


Fig 3.10 Colony PCR using *AOX1* gene primers.
M – 1 Kb Plus DNA Ladder; **Lanes 1-5** – *P.pastoris* recombinants of Pic9K-SRP-I; **Lane 6** – Untranformed *P.pastoris*; **Lane 7** – Pic9K alone; **Lane 8 -** Recombinant with empty Pic9K vector (control); **Lane 9** – Pic9K-SRP-I plasmid.

The PCR products, obtained using *AOX1* promotor and terminator primers should generate two fragments: one corresponding to the size of the SRP gene associated with α-factor – 2167 bp, the other corresponding to the *AOX1* native gene – 2200 bp. Since the difference between gene of interest and native *AOX1* gene was only 33 bp, it was hard to discern these two fragments on the agarose gel. In lane 7, the empty Pic9K vector gives the bright band of approximate 650 bp, which corresponds to α-factor. However, it was interesting that 650 bp band was observed in the transformants too. These obtained results indicated that during transformation into *P.pastoris*, some copies of α-factor were integrated into genome alone, without the SRP gene. This could occur only if in the transforming mixture some empty Pic9K vector remained.

Since the colony PCR using AOX1 promotor and terminator primers showed quite uncertain results, it was decided to use SRP specific primers for colony PCR (Fig 3.11). The obtained results have shown the expected size of fragment – 1490 bp.

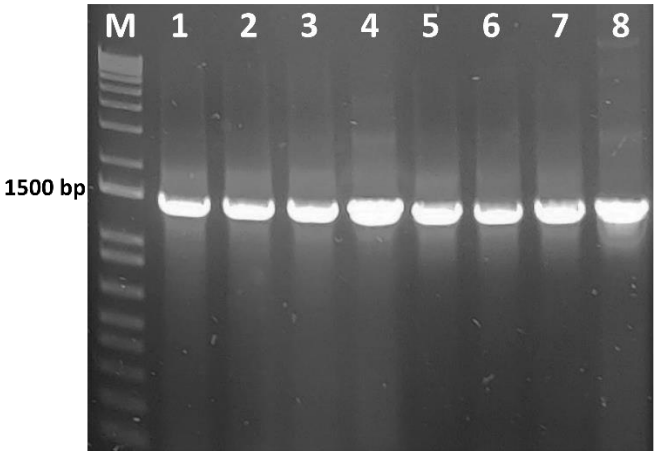


Fig 3.11 Colony PCR using *SRP* gene primers
M - 1 Kb Plus DNA Ladder **Lane 1-7** – Transformants of SRP-I **Lane 8** –Pic9K-SRP-I plasmid.

The transformants with multicopy integration were picked from the plates with the highest concentration of geneticin – 2.0 mg/ml. Further, these clones were used for SRP protein expression.

Expression of SRP protein in P.pastoris

One of the major objectives of this study was to get enzymatically active and extracellularly secreted recombinant protein. After transformation in *P.pastoris*, seven transformants containing *construct I*, the control transformant which contain empty Pic9K plasmid and untransformed yeast were grown in liquid BMGY media (2.2.10) for 18 h at 30˚C shaking at 220 rpm. Further, the cells were harvested, centrifugated and transferred to BMMY media (2.2.10). In the latter media, the cells were grown for 72 h at 30˚C shaking at 220 rpm. Methanol was added into medium every 24 h to a final concentration of 0.5%, in order to maintain constant induction. After cultivation, the supernatant was separated from the cells and concentrated using 30 kDa molecular weight cut-off filters (2.3.13). The cells were disrupted by glass bead method (2.3.14) and fractions of soluble and insoluble proteins

were collected. The samples of intracellular and extracellular proteins were analysed by SDS-PAGE method (2.3.15) (Fig 3.12).

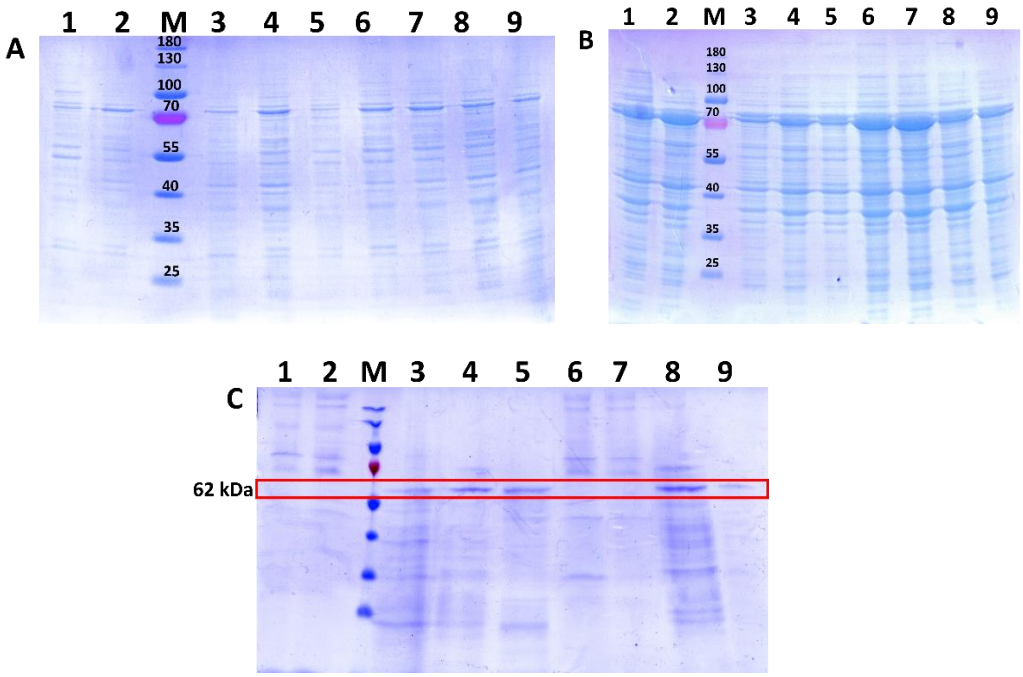


Fig 3.12 SDS-PAGE analysis of recombinant protein expression in *P.pastoris*.
A – fractions of intracellular soluble proteins; B – Fractions of intracellular insoluble proteins; C – Fractions of supernatant proteins; **Lane 1** – untransformed P.pastoris; **Lane 2** – control clones with Pic9K; **Lanes 3-9** – transformants of *construct I*

Analysis of intracellular protein electrophoresis (Fig 3.12 A and B) has not indicated any significant differences between the untransformed clones (Lane 1, Fig 3.12 A and B), control clones with Pic9K (Lane 2, Fig 3.12 A and B) and clones which had the *construct I* (Lane 3-9, Fig 3.12 A and B). Extracellular protein electrophoresis revealed that the SRP is mainly produced as extracellular protein into medium (Fig 3.12 C). The SRP *construct I* should be 62 kDa. In the electrophoresis gel, the 62 kDa band is observed at Lane 4, 5, 6, 8 and 9 in Fig 3.12 C. The untransformed *P.pastoris* (Lane 1, Fig 3.12 C) and Pic9K clones (Lane 2, Fig 3.12 C) have not exhibited any protein band of such size. This indicates that SRP expression was successful and recombinant protein was secreted out into the medium. Further, to investigate the SRP enzymatic activity, the measurement of proteolytic activity was performed (2.3.3) (Table 3.5).

Table 3.5 Determination of enzymatic activity in supernatant fractions after recombinant SRP expression. Relative standard deviation not exceed < 5%.

Supernatant fraction of:	Enzymatic activity, U/ml
Transformant 1 of Pic9K-SRP-I	4.5
Transformant 2 of Pic9K-SRP-I	3.8
Transformant of Pic9K	2.9
Untransformed *P.pastoris*	3.1

The highest enzymatic activity was determined in supernatant fraction of Pic9K-SRP-I transformants 1 and 2 – 4.5 U/ml and 3.8 U/ml, respectively. In supernatant samples of controls, the determined activity was 2.9 U/ml in supernatant of Pic9K transformant; in supernatant of untransformed *P.Pastoris* – 3.1 U/ml. The increased activity in supernatant of transformants with SRP constructs indicated that recombinant protease SRP, which was synthesized into medium, was enzymatically active protease. For higher expression yields, optimization of cultivation conditions is required.

3.4.2 Golden*PiCS* strategy

The second applied cloning approach was focused on the use of the Golden*PiCS* method based on Golden Gate Cloning technique. The Golden*PiCS* plasmid toolkit contains 20 different promoters, 10 transcription terminators, four selection markers and four loci for targeted genome integration. Since the AOX1 promoter is regulated and induced by methanol, which is toxic, this method provides alternative promoters flexible system for the expression of recombinant proteins. The assembly strategy of the cloning system is presented in Fig 3.13.

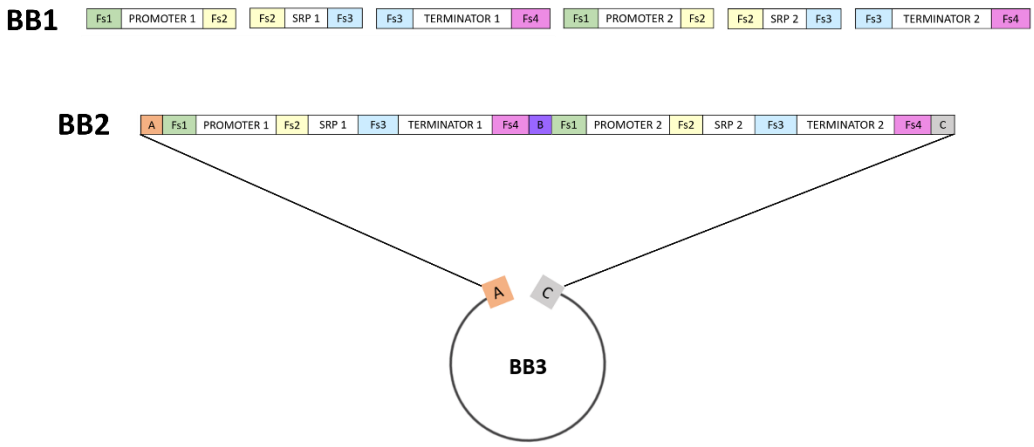


Fig 3.13 The general scheme of Golden Gate Cloning using Golden*PiCS* toolkit. Adapted from Prielhofer et al., 2017.

This system consists of three hierarchical backbone levels for generation of expression plasmids. In the lowest level, individual parts such as promoter, gene of interest and terminator are incorporated into backbone 1 (BB1) plasmids. Subsequently, they are assembled to one transcription unit in BB2 through fusion sites that assure the correct ligation. For the gene of interest fusion sites are added via PCR. Up to eight transcription units can be assembled in BB2. Fusion sites A and C are used for the assembly of BB2 transcription unit into one backbone plasmid BB3, which is designed for subsequent genome integration in *P.pastoris*. This system employs type IIs restriction enzymes that cut outside of their recognition site and enables scarless cloning, and to assemble multiple DNA fragments in one-pot cloning reactions (Prielhofer et al., 2017). The recognitions sites of *Bpi*I are

located in fusion sites region. Therefore, if there are any internal restriction recognition sites within the gene of interest, it must be removed prior cloning.

Two *Bpi*I restriction sites were found in SRP gene sequence. Point mutations in SRP sequence were introduced using multiple site-directed mutagenesis via simple cloning by prolonged overlap extension PCR (POE-PCR) method (Hejlesen & Füchtbauer, 2020). Overlapping PCR fragments were used to create large repetitive multimers, that were further directly transformed into bacteria and are circularized.

For the mutagenesis by POE-PCR, it was decided to use the pUC18 cloning vector (2.2.1) because of its smaller size compared to Pic9K plasmid. The *construct I* was cloned into pUC18 plasmid and further was transformed into *E.coli* DH5α (2.3.8) The pUC18-SRP-I plasmid was extracted using plasmid purification kit (2.3.10). Primer sets were designed, respecting the codon usage of the *P.pastoris* organism, for introduction of point mutations. The PCR was performed using mismatch primers for amplification and pUC18-SRP-I as a template (2.3.6) (Fig 3.14).

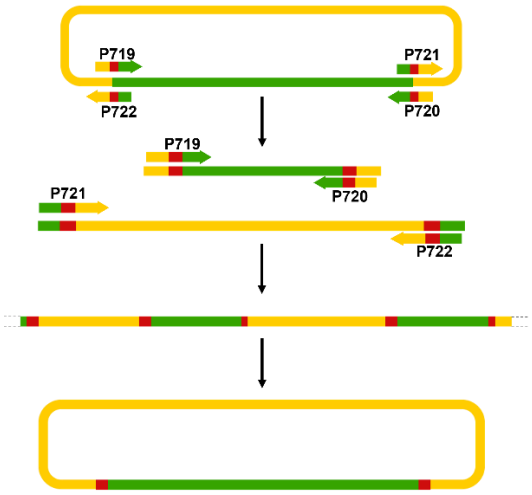


Fig 3.14 The scheme of multiple site-directed mutagenesis using simple cloning by prolonged overlap extension. Red region indicates point mutation. Adapted from Hejlesen & Füchtbauer, 2020.

The amplified DNA fragments contained the overlapping regions between each other. PCR products were purified (2.3.17) and further used for POE-PCR. Long repetitive multimers were obtained and the mixture was directly transformed into *E.coli* DH5α. The mutagenized plasmids were purified (2.3.10) and further analyzed using *Sal*I, *Pag*I, *Mls*I restriction enzymes (2.3.11). The restriction analysis confirmed the presence of SRP construct. The plasmid was sequenced. The results have shown that point mutations were introduced properly and *Bpi*I recognition sites were disrupted. The successfully mutagenized plasmid was further used for Golden Gate assembly.

Golden Gate assembly reaction

GAP promotor, CYC terminator and BB3aZ_14*(2.2.1) backbone plasmid were selected for the construction of expression vector. Each plasmid was transformed into *E.coli* DH5α (2.3.8) and then were extracted using Plasmid purification kit (2.3.10). Additionally, fusion sites were added to

SRP by PCR method, using primers P716/P718 (2.3.6). The PCR product was purified using PCR purification kit (2.3.17). Based on the literature, it was decided to perform the Golden Gate assembly in a different ways.

Initially, the Golden Gate assembly (GGA) (2.3.21) was performed using high concentrations (40nM) of each expression plasmid unit (Prielhofer et al., 2017). However, the effectiveness of transformability was very low, a lot of obtained transformants contained the empty BB3 plasmid. Then, it was decided to use lower concentrations of each unit up to 20fmol (Marillonnet et al., 2020). In addition, to prevent the self-ligation of empty BB3 plasmid with excised part of the vector, restricted backbone vector was used (2.3.11; 2.3.18). Unfortunately, the transformability of such GGA mixture was also very low. After this attempt, it was decided to use circularized BB3 vector and to involve the additional step after GGA reaction, to overcome self-ligation of BB3. This reaction was performed using slightly higher concentration of the constructs – 40 fmol and at after GGA reaction, *Bpi*I was added to restrict the remaining empty backbone vectors (2.3.11). The idea behind the latter step is that only constructed expression vectors should be left in the mixture. The GGA reaction using additional step was successful; a lot of colonies were transformed. In parallel, another GGA reaction was performed and at the end of the assembly reaction, additionally to *Bpi*I, exonuclease *Exo*III was added in order to assure the elimination of empty BB3 vector (2.3.11).

After reactions, the GGA mixtures were directly transformed into *E.coli* DH5α by chemical transformation method (2.3.8). As the backbone vector contains the resistance to zeocin, the transformants were screened using Petri dishes with LB media containing zeocin (2.2.10).

The most promising results were obtained after transformation of GGA reaction mixture when additional steps of restriction were involved. These clones were selected and analyzed performing colony PCR (2.3.9). The P705/P706 primer pair was used to assess the transformants (2.2.2) (Fig 3.15). The PCR product of constructed expression vector should be seen at 2500 bp in length. Empty BB3 plasmid was used as a negative control that gives the fragment of 250 bp.

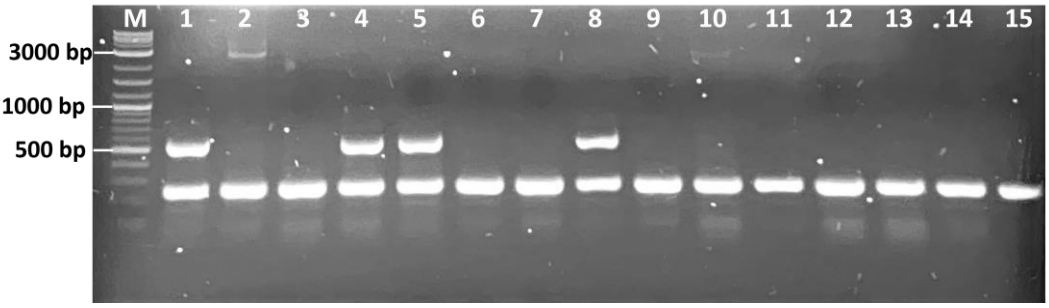


Fig. 3.15 Colony PCR of GGA transformants GGA.

Only two transformants, lane 2 and 10, had the expected fragment of 2500 bp. However, in the same lanes, the PCR product of empty BB3 was amplified too. PCR analysis of other screened transformants revealed that only empty or partially constructed BB3 plasmids were transformed. It is

worth to mention that the observed intensity of the band at 250 bp indicates the high concentration of empty BB3 plasmid.

Transformant 2 was chosen for plasmid extraction (2.3.10). The analysis of plasmid was performed applying *Sgs*I, *Mls*I and *Bsh*TI restriction enzymes (2.3.11). The digestion of applied restriction enzymes revealed that the plasmid was incorrectly constructed. The linearized expression plasmid was expected to be 5000 bp, but restriction analysis identified that it was only 3000 bp in length.

Further, the expression plasmid was sequenced to ascertain the construct. The alignments of sequenced DNA disclosed that it was empty BB3 plasmid without the constructed part of expression cassette. However, such results were ambiguous because the colony PCR had shown the fragment of constructed expression cassette. The possible explanation could be that *Bpi*I and *Exo*I restriction enzymes did not eliminate all of the empty BB3 vector, that further were transformed in the mixture with constructed plasmids. Since both plasmids had the resistance gene for Zeocin, the *E.coli* was capable to maintain both types of plasmids. But the main issue was that the concentration of empty BB3 vector was much higher than the constructed plasmid concentration. Thus, restriction analysis and sequencing had shown the results of empty BB3 plasmid. To obtain the properly constructed plasmids, other clones should be investigated further.

A lot of strategies could be employed for recombinant protein expression, however, simple, flexible and easy to perform methods are preferred. Two cloning methods for development of expression system in *P.pastoris* were applied in this study. Performing the first approach, recombinant protein expression using Pic9K vector was successfully achieved. The enzymatically active SRP protease was secreted into the medium. The obtained results were satisfactory, and the method ensures the precise construction of the expression plasmid, however, it is laborious and time-consuming itself. The second applied approach Golden*Pics* is more flexible and easier to perform system that skips a lot of cloning steps. It provides the capability to apply various different parts of the expression plasmid; however, the transformant screening is complicated and requires further optimization and additional studies in depth.

CONCLUSIONS

1. ATR-FTIR spectroscopy method was developed for the assessment of hydrolysis process.
2. The hydrolysis of alfalfa meal by *Vilzim Pro AD3000* was the most effective at pH 7.0 and 35°C with enzyme concentration of 10% (w/w) for 4 h reaction time.
3. SRP gene was cloned in Pic9K vector and successfully expressed extracellular in *P. pastoris* with the activity of 4.5 U/ml.

VILNIUS UNIVERSITY
LIFE SCIENCES CENTER
Ieva Ožiūnaitė
Master Thesis

DEVELOPMENT OF BIOCATALYSIS BASED TECHNOLOGY FOR THE HYDROLYSIS OF PLANT-DERIVED PROTEINS

SUMMARY

The rapidly growing global population leads to an increased protein demand in food and feed systems. Proteins play a crucial role in supporting essential functions within cells, tissues, organs, and various biological systems. In recent years, the popularity of plant-based proteins has grown due to their benefits over animal-based proteins. Plants provide high levels of proteins without the high content of fats which is often related to animal sourced proteins. Moreover, plant-based proteins are more environmentally friendly than animal-based proteins, requiring less energy and water for production. Protein hydrolysate is the most nutritious form of proteins. It contains a mixture of short-chain peptides and free amino acids. Traditional chemical hydrolysis methods are performed under hazardous conditions that can influence protein structure and functional properties. Oppositely, enzymatic hydrolysis is performed under mild conditions, it is easily controllable due to the specificity of enzymes and environmentally safe method. Protein hydrolysates can be applied in the food industry as functional foods and in the pharmaceutical industry due to the presence of bioactive peptides. These bioactive peptides can exhibit various beneficial features, including antioxidant activity, antihypertensive activity, antimicrobial activity, etc.

In this study, the main aim was to develop a biocatalytic technology for the hydrolysis of plant-based proteins. The study consisted of two parts. The first part was setting the parameters for the hydrolysis of alfalfa meal using commercial enzymes. ATR-FTIR spectroscopy method was developed to assess the hydrolysis process and to determine the degree of hydrolysis (DH). Vilzim Pro AL3000 protease was defined to the best performer and used for further hydrolysis of alfalfa meal. The highest DH was obtained when the reaction was performed under pH 7.0 at 35 °C for 4 h. However, it was observed that during hydrolysis, microbial contamination occurs. The application of antimicrobial agents revealed that sodium nitrite inhibits the growth of microorganisms. The second part of the study was the development of expression system for heterologous recombinant protease in Pichia pastoris yeast. Two different cloning systems were applied for serratopeptidase gene cloning – the conventional cloning method and the Golden*PiCS* approach. The first cloning method was applied successfully, and expression of recombinant protein was achieved. However, for the Golden*PiCS* approach further investigation is required.

DISSEMINATION OF RESULTS

Participation in conferences:

I. Ožiūnaitė, D. Daunoraitė, J. Krutkevičius, I. Matijošytė. Enzymatic hydrolysis of plant-derived proteins. March 1, 2022. The COINS2022. Vilnius, Lithuania. *Poster presentation.*

I. Ožiūnaitė, J. Krutkevičius, I. Matijošytė. Investigation of plant-derived protein hydrolysis via biocatalysis. April 24, 2023. The COINS2023. Vilnius, Lithuania. *Poster presentation.*

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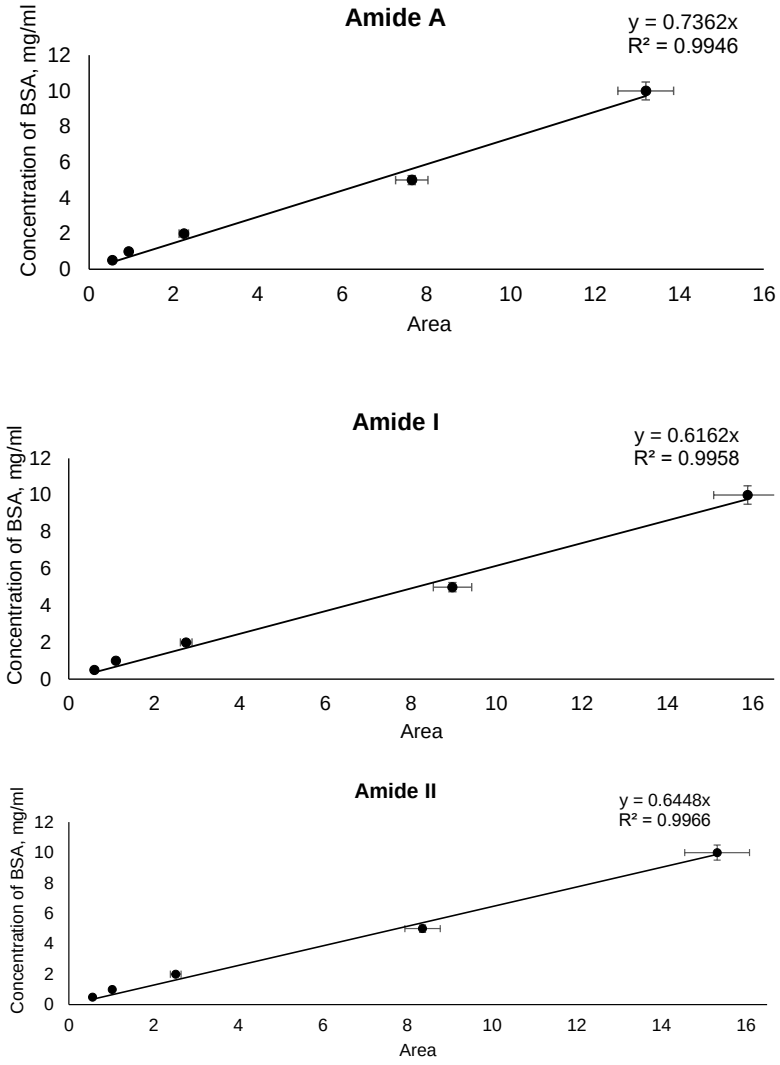
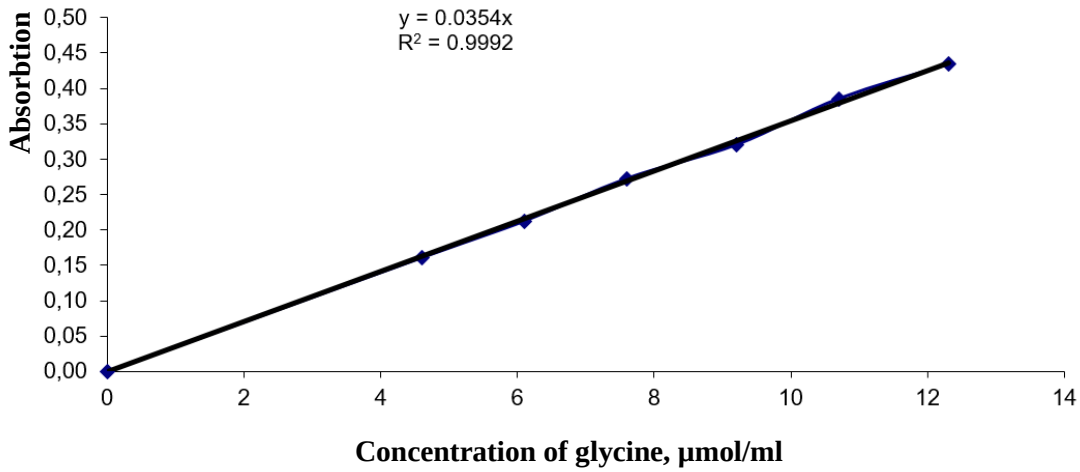
SUPPLEMENTAL MATERIAL



Suplement 1 Calibration curves obtained using the integrated area under the *Amide A*, *Amide I* and *Amide II* bands.



Suplement 2 The calibration curve of glycine for protease activity measurement using N,N-dimethylcasein**.**

	Circularized vector (40 mM) 800 fmol				**Restricted vector 20 fmol**				**Circularized vector 40 fmol**			
Reaction	1	2	3	4	5	6	7	8	9	10	11	12
BB3aZ_14* (restricted)	-	-	-	-	X	X	X	X	-	-	-	-
BB3aZ_14* (circularized)	X	X	X	X	-	-	-	-	X	X	X	X
GAP promotor	X	-	-	-	X	-	-	-	X	X	-	-
CYC terminator	X	-	-	-	X	-	-	-	X	X	-	-
DNR of interest (*SRP* gene)	X	-	-	-	X	-	-	-	X	X	-	-
FastDigest buffer	2	2	2	2	2	2	2	2	2	2	2	2
ATP	2	2	-	-	2	2	-	-	2	2	2	-
T4 DNR ligase	1	1	-	-	1	1	-	-	1	1	1	-
*Bpi*I	1	1	1	-	1	1	1	-	1	1	1	-
H_2O	Up to 20	Up to 20	Up to 20	Up to 20	Up to 20	Up to 20	Up to 20	Up to 20	Up to 20	Up to 20	Up to 20	Up to 20
*Bpi*I (Additional step)	-	-	-	-	-	-	-	-	0,5	0,5	-	-
Exo III(Additional step)	-	-	-	-	-	-	-	-	-	0,5	-	-

X – amount of DNR is estimated by formula: Y (fmol) × lenght (bp) / concentration (ng/µl) / 1520
Y – 40 or 800 fmol (concentration of 40mM)

Suplement 3 Composition of the reactions of Golden Gate assembly (µl).