DIPLOMA THESIS

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REDUCTION OF MILK PROTEIN ALLERGEN BY PHYSICAL AND ENZYMATIC METHODS

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1 Introduction

Prior to the early 1900s, providing newborns with artificial food was risky and led to a high mortality rate. The introduction of pasteurization and the recognition of the significance of using clean water in cow milk improved the safety of artificial feeding. However, even with these precautions, some infants failed to thrive on cow milk-based formulas, experiencing severed gastrointestinal symptoms were linked to the foreign proteins present in cow milk. Nevertheless, due to the substantial nutritional value and widespread availability of cow milk proteins, they have continued to be the primary protein source for infants in developed Western countries. The allergic nature of cow milk proteins has consistently raised concerns within the realm of infant nutrition (Savilahti, E., 1992).

Presently, the researchers are prompted to discover functional foods that promote consumers' overall health. The International Dairy Federation (IDF) has emphasized the numerous advantageous qualities of proteins found in cow's milk. This bears favourable consequences for the functional food industry.

Cow's milk protein allergy (CMPA) affects from 2 to 6 % of children, with the highest prevalence during the first year of age (Hill and Hosking, 1996, 1997; Hosking et al., 2000). According to the study by Host, (2002), the occurrence of CMPA in infancy appears to be around 2 to 3% in developed nations. About 5 to 15% of infants might experience symptoms suggestive of CMPA, underscoring the significance of employing controlled elimination and milk challenge approaches. Replicable clinical reactions to CMP within breastfeed infants are noted in roughly 0.5% of cases. Most CMPA symptoms manifest before one month of age, typically within a week of introducing CMP-based formula. These symptoms might emerge either within an hour of milk intake (immediate reactions) or after one hour (delayed reactions). The prognosis of CMPA is favourable, with remission rates of about 45 to 50% at one year, 60 to 75% at two years, and 85 to 90% at three years.

The allergenic or antigenic nature of a protein is influenced by its primary structure (amino acid sequence and attached carbohydrates) and its molecular conformation. The primary structure supplies elements for antibody binding, while the protein's conformation dictates the specific elements engaged in epitopes, the antigen-binding sites. Consequently, modifying either the primary structure or the protein's conformation can lead to a reduction in allergenicity or antigenicity (Lee, 1992). Enzymatic hydrolysis is viewed as the optimal approach to diminish

the allergenic reactivity of CMPs. The manipulation of milk protein hydrolysis can be regulated through the choice of specific enzymes, operating conditions, and parameters. Proteolytic enzymes, like papain from papaya latex (EC 3.4.22.2), bromelain from pineapple stems (EC 3.4.22.32) and fruits (EC 3.4.22.33), ficain (EC 3.4.22.3) from fig latex, zingibain (EC 3.4.22.67) from ginger, actinidin (EC 3.4.22.14) from kiwi fruit are employed in food applications due to their non-induction of unwanted sensory properties (Ha et al., 2012).

Previous research has confirmed that utilizing trypsin (a serine protease) for protein hydrolysis can effectively lower the allergenic potential for proteins in cow's milk (Nath et al., 2020).

The primary focus of cysteine protease studies has revolved around papain, the most representative enzyme of this class. Papain is a globular protein composed of 212-residue polypeptide chain, folded to shape two domains separated by a deep cleft. The active site's cysteine residue (Cys-25) is situated within the L1 R-helix on the left domain's surface, while the histidine (His-159) resides in a sheet on the right domain's surface. These two ionizable groups, acting as crucial catalytic components, align with papain's pH-dependency (Harrison et al., 1997).

Diverse physical techniques have been utilized for producing allergen-free foods, including Ultra Heat Treatment (UHT), High-hydrostatic pressure treatment, high-pressure homogenization, and Ultrasound treatment. However, several drawbacks of physical methods have been published. In many cases, physical treatment causes the secondary structure of the protein to break down and hidden epitopes are exposed (Abbas Syed et al., 2019).

Ohmic and radiofrequency treatment also cause allergenicity level reduction. The present study deals with studying the enzymatic hydrolysis of papain and the combination of the recently mentioned heating alternatives with enzymatic hydrolysis.

2 The goal of the thesis work

Since cow's milk contains almost all the essential amino acids, it serves as a valuable nutrient intake. Nevertheless, it is also important to note that proteins present in cow's milk are part of the group of eight most frequently encountered allergens.

Initial studies indicate that the inclusion of a protease enzyme can diminish the presence of the problematic whey protein, thereby mitigating allergenic properties.

In the present era, there is an increased focus on consuming allergen-free diets, leading to a rising request for such products.

Numerous drawbacks associated with the physical methods employs in manufacturing allergen free items have been documented. For instance, due to the application of physical processes, the protein's secondary structure may deteriorate, causing concealed epitopes to become exposed.

Enzymatic and acidic treatments were evaluated as effective approaches for decreasing allergenic properties. However, acid treatment in the case of plant proteins can lead to the formation of harmful substances such as 1,3-dichloro-2-propanol (1,3-DCP) and 3-monochloro-1,2-propane-diol (3-MCPD), which makes it unsuitable for use in food processing due to the potential toxicity (Kim et al., 2015). On the other hand, enzymatic hydrolysis is regarded as the optimal method for reducing allergenicity.

Through the careful choice of a particular enzyme and the fine-tuning of hydrolysis conditions (enzyme concentration, hydrolysis time and temperature), the level of allergenicity of cow's milk can be reduced to a large percentage.

Several studies have already investigated the ability of trypsin to reduce allergenicity, with desirable results. Trypsin of animal origin, which produced in the pancreas in humans, can be produced artificially with microorganisms. In contrast, papain can be extracted from papaya latex.

The objective of this work was to enhance the efficiency of the papain enzyme with the intention of establishing a foundation for developing a product devoid of milk protein allergens. Additionally, the refined conditions were integrated with various methods of heat applications to assess their impact on the degree of allergenicity.

3 Literature overview

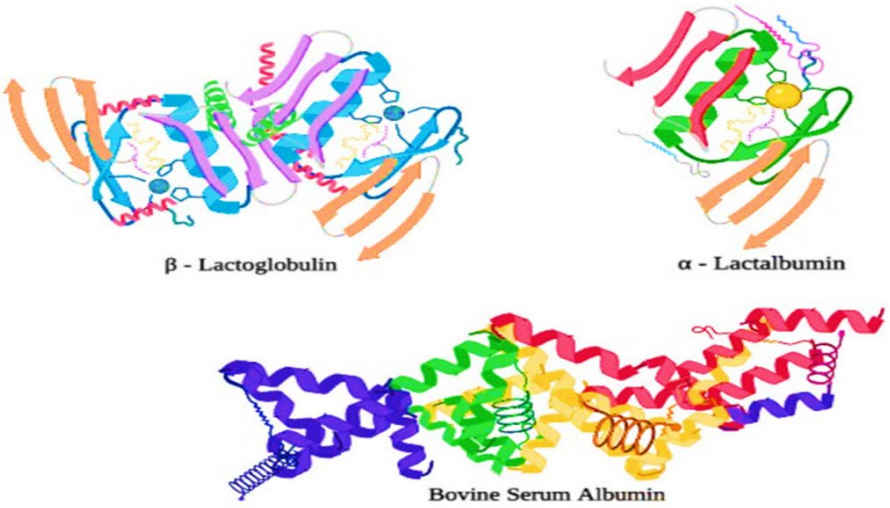
3.1 Unprocessed cow's milk

Raw cow's milk possesses over three times the protein concentration found in human milk. In milk, about 33 gm/L^2, nearly 80% of the group of proteins belongs to the caseins. The shared characteristic among caseins is their limited solubility at a pH of 4.6. At this specific pH, the whey proteins remain dissolved. Whey proteins constitute 19% of the total protein content in a raw cow's milk (6.3 grams per litre). The predominant whey protein, known as β-lactoglobulin, naturally exists as a dimer formed by two individual subunits. There are at least six known genetic variations of β-lactoglobulin. Another whey protein, α-lactalbumin, has two genetic forms that differ by only a single amino acid. Interestingly, the amino acid sequence of α-lactalbumin is identical to that of lysozymes. Its biological function facilities the conversion of galactose to glucose in order to create lactose.

Bovine serum albumin is not produced within the mammary gland. Instead, it enters the milk through secretory cells, constituting approximately 1.2% of the overall milk protein content. The whey holds various other components, including orosomucoid and folate-binding protein. Roughly 1% of the total protein in cow's milk is found in the fat globules, and these globules can be fractionated from solubilized membranes. Furthermore, trace amounts of β_2-microglobulin are present in cow's milk (2 mg/L). Two iron-binding proteins exist within cow's milk: transferrin, which resembles the variant found in blood serum, and lactoferrin, generated within the mammary gland. The concentration of transferrin in fully developed milk reaches approximately 100 milligrams per litre. Cow's milk also contains immunoglobulins, constituting 2.1% of the total protein content in mature milk. Unlike human milk, the primary immunoglobulin present in cow's milk is IgG1, which is abundantly transferred to the colostrum. Like human milk, cow's milk has secretory IgA, where two individual IgA molecules are connected by a J chain and the secretory component. The IgM takes the form of a pentamer connected by J chains and the secretory part, like its structure in human milk (Savilahti and Kuitunen, 1992).

3.2 Allergenicity

Despite its many beneficial properties, however, cow's milk has proteins (allergens) that are considered antigens in milk and can trigger an allergic reaction. They used a response surface methodology (RSM) to optimise the reaction conditions (Savilahti and Kuitunen, 1992).



1. Figure The polymeric structure of whey proteins and their fractions (Krishna et al., 2021)

3.2.1 Epitopes

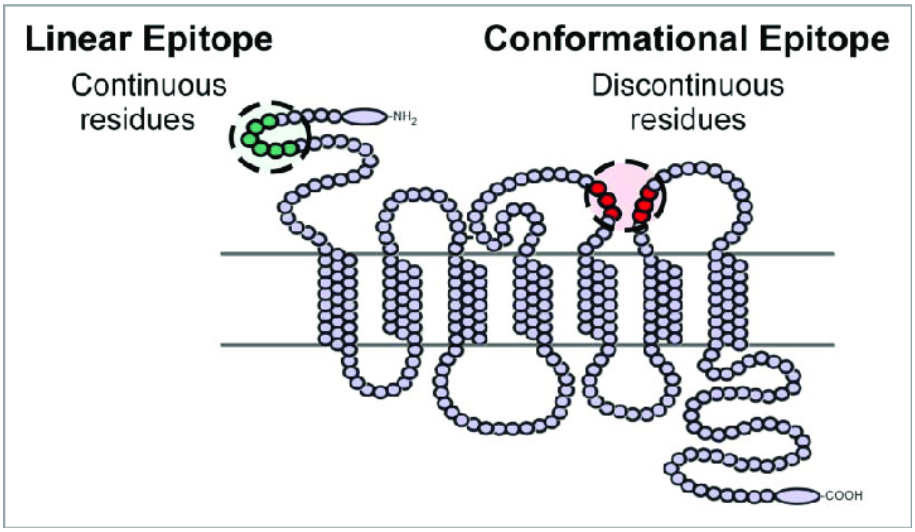
In the review by Kormosné Bugyi, 2012, is mentioned that the immunogenicity of a protein is basically determined by its amino acid sequence and 3D structure. The binding of allergenic protein to specific IgE antibodies can occur under a combination of conditions. Firstly, the protein must be sufficient size to form a bridge between two IgE molecules, secondly, it must have more than one IgE-binding site. The latter must be sufficiently far apart to form a bridge between the two antibodies.

The immunoreactive part of a protein molecule that is recognised by antibodies, is called the epitope. We can talk about conformational or sequential (or linear) epitopes. The sequential epitopes are formed only by the primary structure of the protein and form an integral part of the protein chain, making them largely heat stable. Conformational epitopes are formed by

juxtaposition of molecular parts in the amino acid sequence. Consequently, the structure of the molecule (Jackson, 2003 and Mills et al., 2005).

Individuals in whom the IgE-mediated reaction is triggered by a sequential epitope, all forms of food (raw or processed) trigger the onset of symptoms, whereas those in whom the IgE-mediated reaction is triggered by a conformational epitope are well tolerate heat-treated or partially hydrolysed foods well, as the protein epitope is destroyed by changes in the protein structure. It has also been shown that the specific reactivity of IgE-binding epitopes correlates better with clinical reactivity than the quantitative IgE levels elicited by total protein. The epitopes determination can also help to estimate the severity if an allergic reaction, as it has been shown that the IgE that patients whose IgE antibodies react with more than one epitope have a more severe symptom (Sampson, 2004).

In prior research, IgE-binding epitopes for various milk proteins, including α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, α-lactalbumin, and β-lactoglobulin, were identified using a method involving the synthesis of overlapping decapeptides with the SPOTs membrane provided by Genosys Biotechnologies, Inc. These studies aimed to compare the sequential epitope recognition between older patients with persistent cow's milk allergy and younger children who were likely to outgrow their CMA due to decreasing milk specific IgE antibody levels. A total of 25 epitopes were found across different cow's milk proteins: 5 from α_{s1}-casein, 5 from α_{s2}-casein, 9 from κ-casein, 1 from α-lactalbumin, and 5 from β-lactoglobulin. These epitopes were specifically recognized by IgE antibodies from children with persistent CMA but not by IgE from individuals expected to outgrow their allergy (Järvinen et al., 2002).



2. Figure Linear and Conformational Epitopes (Nath, 2022)

3.2.2 Antigenicity and allergenicity

Cow milk proteins can trigger an immune response, either involving antibodies (humoral) or immune cells (cell mediated), or even a combination of both, once they have crossed the protective barrier of the gastrointestinal tract. These protein components that evoke immune reactions are composed of 11 to 13 amino acids and are presented to B cells along with class II major histocompatibility complex molecules. Recently, the amino acid sequence of a peptide from Bovine Serum Albumin (BSA) bound to HLA class II molecules was elucidated.

For protein antigens, a collaborative interaction between T helper cells and B cells is necessary to initiate the immune response. When an infant consumes cow milk, their immune system always responds to this protein, leading to the production of IgG antibodies circulating in the bloodstream and IgA antibodies in the mucosal tissues. This immune response development could potentially have a protective and anti-inflammatory function. However, if an infant experiences negative clinical symptom upon consuming cow milk proteins and simultaneous exhibits an abnormal immune response to these proteins, they are considered allergic to cow milk.

The nature of the immune response, whether protective or allergic, seems to be influence by the specific type of T helper cells activated by the antigen. In mice, two distinct types of T helper cells were initially named based on their secretory profiles for lymphokine release. T helper 2 (Th2) cells secrete interleukin-4 without interferon gamma, leading to heightened IgE secretion by B cells. On the other hand, Th1 cells secrete lymphokines that prompt B cells to secrete IgG and IgA. In humans, T-cell clones from atopic patients, stimulated by allergens like house dust mites, exhibit a secretory pattern similar to Th2 cells in mice, inducing IgE secretion by B cells in vitro. Moreover, allergic reactions can be triggered not only through IgE-mediated responses but also by cell-mediated immunity. However, stimulation of CD4+ cell clones specific to substances like nickel can lead to Th1-like secretory pattern, both in patients with contact dermatitis and non-allergic individuals.

In mice, the type of major histocompatibility complex presenting the antigen plays a role in determining the type of helper cells that become activated. Additionally, the lymphokines secreted by Th2 cells can stimulate the proliferation of precursor cells for various types of mast cells. The IgE is bound to specific receptors on these cells as well as on eosinophils, macrophages, and platelets. When an allergen binds to two molecules of specific

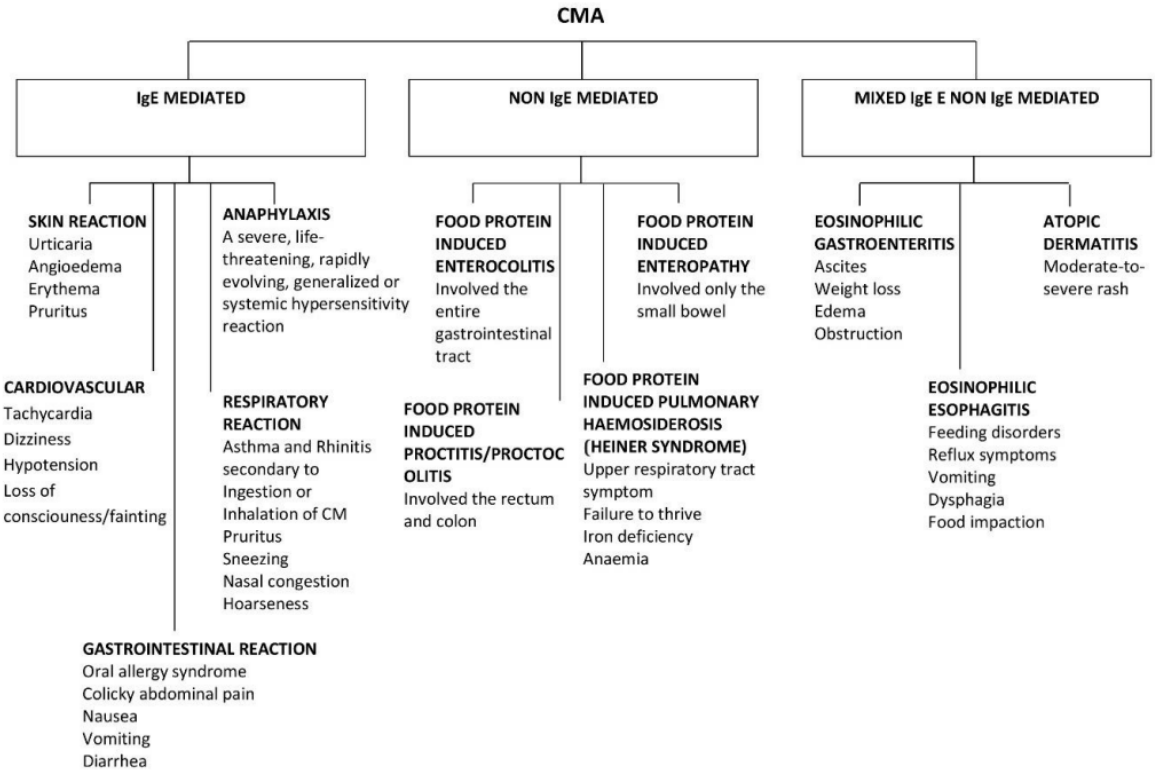
IgE fixed on these cells, an immediate hypersensitivity reaction is triggered, releasing potent inflammatory mediators. The mechanisms underlying delayed allergic reactions are less understood. Infants with delayed reactions to cow milk protein exhibit changes in their intestinal structure, including crypt hyperplasia, villous atrophy, increased lymphocyte density in the epithelium, and infiltration of mononuclear cells in the lamina propria. The density of IgA- and IgM-containing cells in the intestine significantly increases during clinical relapse. Both immune complex-mediated (Arthus reaction) and delayed, cell-mediated reactions alight with these observations (Savilahti and Kuitunen, 1992).

3.2.3 Oral tolerance

Numerous laboratory animals demonstrate a form of tolerance (an alternate immune response) to protein antigens introduced through ingestion. This can lead to a state of overall unresponsiveness in both the humoral and cell-mediated aspects of the immune system, which can be induced by protein antigens consumed during feeding. Whether an animal develops tolerance or initiates an immune response depends on factors like the specific type of animal, the timing of exposure, and the quantity of protein ingested. An animal exhibiting tolerance does not exhibit a secretory immune response in the mucosal tissues either. However, the occurrence of oral tolerance hasn't been observed in humans.

The reactivity of the immune system to cow milk proteins diminishes as an individual grows older. The levels of circulating IgG antibodies specific to cow milk begin to decrease after the first year of life, while IgA antibodies gradually decrease later in childhood. The majority of patients suffering from cow milk allergy eventually become clinically tolerant to cow milk during their childhood. The type of allergy characterized by delayed intestinal symptoms typically disappears, often before the age of two. On the other hand, IgE-mediated cow milk allergy, which predominantly results in skin symptoms, fades away at a slower pace. Even patients who have developed clinical tolerance to cow milk might still show circulating cow milk specific IgE antibodies and show positive reactions in skin prick tests.

The emergence of clinical tolerance might be associated with the maturation of the intestine and its enhanced capacity to effectively prevent the passage of antigens from ingested food (Savilahti and Kuitunen, 1992).



3. Figure Clinical presentation of IgE and non-IgE cow's milk allergenicity (Giannetti et al., 2021)

3.2.4 Cow's milk allergy versus cow milk intolerance

The consumption of cow's milk can lead to various risks, either due to contamination with harmful agents or due to disturbances in digestion, absorption, metabolisms or immune response in individuals. Recurrent or chronic adverse reactions to cow's milk are colloquially labelled as cow's milk intolerance (CMI) or cow's milk allergy (CMA). Although these two terms differ both in linguistic and scientific contexts, they are often used interchangeably by both the general public and healthcare practitioners.

„Allergy" has traditionally been associated with hypersensitivity, denoting a clinical reaction mediated by the immune system. As per the Webster's Unabridged Dictionary, allergy describes a „condition of heightened sensitivity to substance that doesn't affect others in similar amounts." On the other hand, „intolerance" indicates an „inability to tolerate", essentially being a broad, nonspecific term. CMI typically results from inadequate digestion, often of the milk

sugar lactose and occasionally of its fat content. It can also stem from specific inborn metabolic error linked to certain milk constituents or their metabolites, such as in cases of phenylketonuria and galactosemia. While not true intolerance in the strict sense, aversions and psychological reactions to milk ingestion are sometimes referred to as such, primarily by the public.
Bahna's (2002) study centres on a comparison between cow's milk allergy (CMA) and lactase deficiency (LD), which recognized as the foremost contributor to cow's milk intolerance (CMI) on a global scale.

3.2.4.1 Cow Milk Allergy

Cow's Milk Allergy (CMA), a primarily childhood disease with varying prevalence estimates due to factors like age, feeding habits, and diagnostic criteria. CMA is influenced by proteins in cow's milk, particularly casein and whey. Sensitivity varies among individuals, and even small amounts of these proteins can cause sensitization in utero or through nursing. CMA reactions can be immediate (Type I) or delayed (Type IV) immune responses, with Type I being the most common and related to IgE antibodies. Diagnosis involves medical history, physical testing, serum IgE levels, and specific allergen testing. Management involves strict milk avoidance, using milk substitutes, but allergic reactions to some substituents can occur. CMA prognosis is generally temporary in young children with milk avoidance, with tolerance developing over time, and gastrointestinal symptoms often improving faster than other allergic manifestations. Severe reactions can last for many years (Bahna, 2002).

3.2.4.2 Lactose intolerance (LI)

Lactose is the exclusive carbohydrate in mammalian milk and absent in other foods. Its content varies, with the highest levels in human milk, followed by bovine and goat milk, and lowest in seal and whale milk. Lactose maldigestion results from inadequate intestinal lactase enzyme relative to lactose intake, leading to gastrointestinal symptoms.

The enzyme lactase breaks lactose into absorbable glucose and galactose in the small intestine. Unhydrolyzed lactose in the colon ferments, causing symptoms like bloating, flatulence, abdominal pain, or frothy diarrhoea. The severity depends on lactose intake and intestinal lactase activity.

Lactose intolerance (LI) forms include congenital, secondary due to small intestine disorders, and primary acquired. Diagnosis involves symptoms after milk consumption, stool acidity, and reducing substances. Laboratory tests include lactose tolerance, breath hydrogen, and lactase activity measurement in a mucosal biopsy.

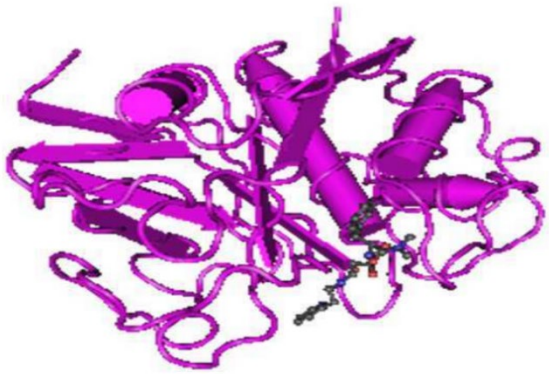
Management involves reducing lactose intake to control symptoms, except in congenital cases. Lactose-free formulas are used for infants, while secondary LI requires lactose avoidance post-recovery. Delayed-onset LI can persist but can often tolerate some milk or specific products. Lactase enzyme in yogurt and aged cheeses can aid digestion. Lactase preparations, both oral and added to milk, are available to aid digestion. Commercial lactose-reduced milk products are also accessible (Bahna, 2002).

3.3 Plant based protease

3.3.1 Papain

Papain (EC 3.4.22.2) is an endolytic enzyme derived from the latex of the papaya plant (Carica papaya L.), categorized as a plant cysteine protease. This enzyme is obtained by making an incision in the skin of unripe papaya and collecting the latex that emerges from the cut, which is then dried. The potency of papain increases with the level of fruit ripeness, with greener fruits yielding more active enzyme content. Papain belongs to the papain superfamily and plays as a vital role as a proteolytic enzyme in various essential biological processes across all living organisms, as indicated by Tsuge et al. (1999). Papain demonstrates significant proteolytic activity, targeting proteins, short-chain peptides, amino acid esters, and amide bonds. Its applications span the fields of both food and medicine, as detailed by Uhlig (1998).

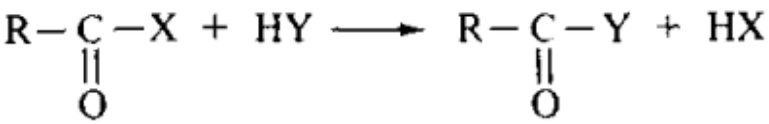
Amri and Mamboya's (2012) review contends that papain has been identified as an enzymatic protein with substantial significance in both biological and economic contexts. The distinctive structure of papain contributes to its functionality, aiding in the comprehension of its proteolytic mechanisms and enhancing its value for diverse applications. It is imperative to conduct further investigations into the papain enzyme to comprehend its specificity, structural attributes, and the impacts it induces on various thermodynamic pathways. Papain is naturally present in papaya, a versatile plant with multiple uses (e.g., drug design, industrial uses and pharmaceutical preparations, medical uses) and enzymatic characteristics. Given the wide range of climates in which papaya thrives, cultivating papaya to extract papain has the potential to generate significant income for farmers.



4. Figure Papain structure (Amri and Mamboya, 2012)

The latex in the fruit of the tropical papaya tree (Carica papaya) contains many enzymes. One of the favourable characteristics of dried latex is its proteolytic property, which allows it to be used in many industrial applications. For this purpose, the dried latex is sold as „papain" or „crude papain". It contains at least two different proteolytic enzymes: chymopapain and papain. Over the years, many studies have been carried out on chemical, physical and kinetic studies. The results of these studies have provided a fairly comprehensive picture of the structure of the enzyme. The sequence of amino acid residues and their relative positions in the molecular structure are now known, but a detailed picture of the enzymatic mechanism is not yet available. Although it is known that chymopapain is the main component of raw papaya latex, few studies have so far been directed towards the enzyme, probably due to the heterogeneous nature of its preparation. Both enzymes belong to the group of proteolytic plant enzymes and require a sulfhydryl group to function. The catalytic activity of the sulfhydryl proteinases is easily destroyed by chemical compounds known to react with a sulfhydryl group, e.g., metal compounds, oxidizing agents, alkylating agents, and disulfides (Drenth et al, 1971).

Besides the hydrolysis of peptide bonds, papain can also catalyse several other reaction types. These reactions can be represented by the following equation:



5. Figure Basic equation for the hydrolysis of papain (Drenth et al., 1971)

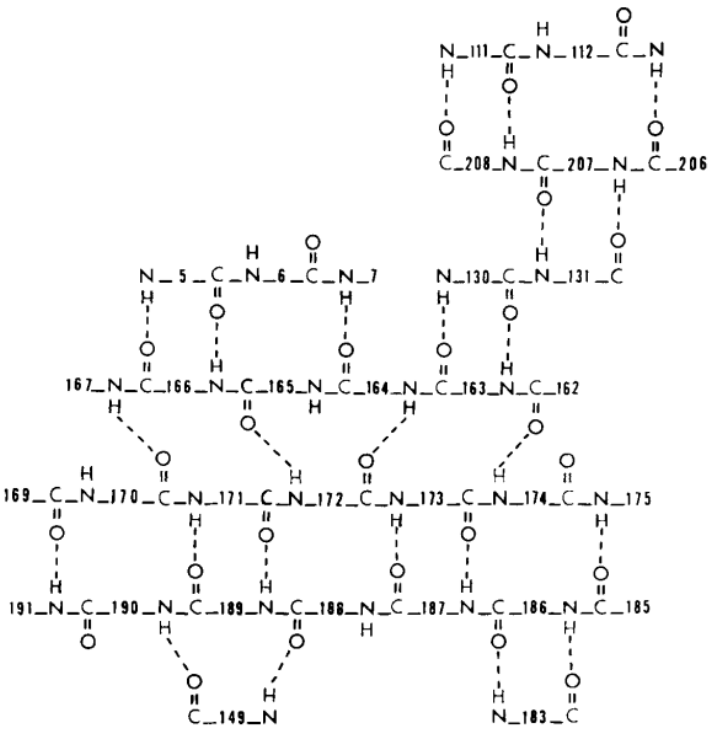
3.3.1.1 Properties and Structure of Papain

The papain enzyme is a globular protein characterized by a single chain. It has a molecular weight of 23,406 Da and comprises 212 amino acids, featuring four disulfide bridges and catalytically significant residues at positions Gln19, Cys25, His158, and His159 (Mitchel et al., 1970; Robert et al., 1974; Tsuge et al., 1999). The amino acid composition of papain remains stable and functional across a wide range of conditions, even at elevated temperatures (Cohen et al., 1986). It remarkably maintains stability even in the presence of strong denaturing agents such as 8M urea organic solvents like 70% EtOH.

Papain's optimal pH for activity spans from 3.0 to 9.0, although this range varies based on different substrates (Edwin and Jagannadham, 2000; Ghosh, 2005). As part of the papain superfamily of cysteine proteases, the papain enzyme typically consists of two well-defined domains, offering an excellent model for studying protein folding and unfolding behaviour (Edwin et al., 2002). Stabilized by three disulfide bridges, the enzyme's molecule is folded along these bridges, creating strong interactions among side chains that contribute to its stability (Edwin and Jagannadham, 2000; Tsuge et al., 1999).

The three-dimensional structure of papain comprises two distinct structural domains with a cleft between them. This cleft houses the active site, including a catalytic dyad analogous to the catalytic triad found in chymotrypsin. The catalytic dyad consists of the amino acids' cysteine-25 (from which it derives its classification) and histidine-159. Initially, aspartate-158 was believed to play a role similar to that of aspartate in the catalytic triad of serine proteases, but this notion has since been disproven (Menard et al., 1990).

Papain's molecule features an all-α domain and an antiparallel β-sheet domain (Kamphuis et al., 1984; Madej et al., 2012). Its conformational behaviour has been studied in aqueous solutions containing SDS, revealing a high α-helical content. When in the presence of SDS, papain's structure unfolds due to strong electrostatic repulsion (Huet et al., 2006). In the molten globule state at pH 2.0, papain retains substantial secondary structure in the form of β-sheets and is comparatively less denaturised compared to being exposed to 6 M Guanidium Hydrochloride (GnHCl). Furthermore, the enzyme demonstrates a tendency to aggregate at lower concentrations (Edwin and Jagannadham, 2000).



6. Figure Residues involved in the twisted pleated sheet structure occurring in the papain molecule (Drenth et al., 1971)

3.4 Allergenicity reduction technologies

The study by Morisawa et al., 2009, investigated the effects of heat treatment and enzymatic digestion on B cell epitopes of milk proteins using a histamine assay with cultured human basophils sensitized with patient sera. Unlike using peripheral blood basophils due to practical limitations, cultured basophils were employed for assay standardization. Heat treatment induced intermolecular disulfide bond formation and conformational changes in β-lactoglobulin, resulting in reduced histamine release in response to this protein. Enzymatic digestion was facilitated by heat treatment, contributing to decreased allergenicity. However, some patients showed persistent allergenicity even after treatment. Heat treatment did not alter α-casein's molecular weight, and its allergenicity. These finding explained the variable clinical responses to heat-treated milk and suggested the diagnostic value of heat-treated and/or digested protein assays for food hypersensitivity, especially involving IgE-specific responses. The presence of disulfide bonds might influence allergen persistence, indicating a possible marker for persistent symptoms.

3.4.1 Physical treatments

3.4.1.1 Ultra-Heat Treatment

Heating plays a crucial role in the production of dairy products, causing structural and chemical changes in proteins like denaturation, aggregation and Maillard reactions. These transformations can significantly impact the allergenicity of milk protein allergens.

Among cow's milk proteins, casein is highly heat-resistant, while whey proteins are more sensitive, following the order: immunoglobulins (Ig), bovine serum albumin (BSA), β-lactoglobulin (β-LG), α-lactoglobulin (α-LA). Heating at 120 °C for 15 minutes does not affect bovine casein's antigenicity, but BSA and immunoglobulins lose their antigenic properties at temperatures ranging from 70 to 100 °C. Studies have indicated that heat treatment can lead to changes in IgE-binding capacity with various proteins, with decrease in IgE binding noted for β-LG and BSA after heat treatment.

Research by Bu et al., (2009) explored the impact of heat on α-LA and β-LG increased between 50 to 90 °C but significantly decreased beyond 90 °C. This rise in antigenicity might be due to the exposure of previously concealed allergenic epitopes during heat denaturation. Conversely, the decline in antigenicity at temperatures over 90 °C could be attributed to the destruction or masking of epitopes by chemical reactions and aggregation processes. More severe heating conditions might also lead to the Maillard reaction, causing a loss of linear epitopes and reduced antigenic response.

In vivo studies have also investigated the effect of heating on milk protein allergenicity. Heat-denatured β-LG induced stronger local immune responses in the gastrointestinal mucosa of rats compared to native β-LG. High heat (baking) has been shown to reduce the allergenicity of food proteins by altering their conformation and destroying allergenic epitopes.

It's important to note that the alteration of milk protein allergens through thermal processing depends on factors like milk composition, processing conditions, exposure circumstances, and individual genetic makeup. Therefore, careful control of thermal processing conditions is necessary when developing hypoallergenic milk products (Bu et al., 2013).

In the context of milk, denaturation of whey proteins is primarily driven by β-LG, which constitutes around 50% of the whey protein content. However, α-LA also plays a notable role in the denaturation process (Krishna et al., 2021).

3.4.1.2 High hydrostatic pressure technology

High-pressure treatment also amplifies the pepsin-driven hydrolysis of β-LG at 400 MPa, resulting in a decrease in the antigenicity and the binding of IgE to β-LG. This suggests the potential to generate hypoallergenic β-LG hydrolysates (Chicon et al., 2008). Lactoglobulin denaturation occurs with a pressure treatment of 500 MPa at 25 °C. Notably, denaturation of immunoglobulins and lactalbumins takes place primarily at the highest pressures, especially at temperatures surpassing 50 °C. This highlights the preservation potential for colostrum immunoglobulins, which are susceptible to damage during heat treatment (Felipe et al., 1997).

Liu et al., (2005) investigated the impact of high-pressure processing on the hydrophobicity of whey protein concentrate and found that applying high pressure led to an increase in the number of binding sites. This induced certain alterations in the proteins, enhancing their hydrophobicity and offering promising outcomes for enhancing the functional attributes of food products. Similar positive findings were reported for improved properties such as hardness, surface hydrophobicity, solubility, gelation, and emulsifying capabilities in whey protein functionality (Lee et al., 2006).

High hydrostatic pressure (HHP) treatment offers numerous advantages compared to conventional processes. It is swift and ensures uniform pressure distribution, regardless of sample size and shape, minimizing thermal degradation (Chawla et al., 2010).

3.4.1.3 High pressure homogenisation

High pressure constitutes a novel approach within the realm of food processing techniques. When applied to food-high pressure treatment has the capability to induce changes in the structure of milk proteins, such as denaturation and the formation of aggregates. These alterations potentially impact the allergenic nature of milk proteins (Bu et al., 2013).

Kleber and colleagues (2007) revealed that subjecting substances like β-lactoglobulin (β-LG) in solutions containing whey protein isolate (WPI), sweet whey, and skim milk to high-pressure conditions ranging from 200 to 600 megapascals (MPa) across temperatures from 30 to 68 degrees Celsius could heighten the antigenic properties of β-LG. Similarly, another investigation indicated that subjecting β-LG and WPI to high-pressure treatment at 200 and 400 MPa led to an increase in their binding to specific antibodies without affecting their binding to IgE antibodies from allergic patients. This rise in antigenicity is thought to stem from the exposure of epitopes usually hidden within the native protein structure. These previously inaccessible regions become available for antibody binding due to the unfolding and aggregation caused by pressure-induced effects.

In parallel, the changes in protein conformation caused by high pressure might facilitate enzymatic breakdown. β-lactoglobulin can be effectively broken down by various enzymes under hight-pressure conditions. These hydrolysates, generated through enzymatic action on β-LG under high pressure, could exhibit diminished antigenicity and reduced binding to IgE antibodies. Notably, the impact of high-pressure treatment (ranging from 100 to 300 MPa) on the enzymatic hydrolysis of dairy whey proteins by enzymes like trypsin, chymotrypsin, and pepsin was examined. The results indicated that high pressure enhanced protein hydrolysis and decreased the residual antigenicity of the hydrolysates, with the extent of reduction depending on the specific enzyme used.

Recent work by López-Expósito and colleagues (2012) focused on evaluating the allergenic potential of β-LG hydrolysates created using chymotrypsin under both atmospheric and high-pressure conditions. The research employed a mouse model of β-LG allergy and found that the hydrolysates tested lost their allergenic properties, as indicated by the absence of allergic reactions and a drop in body temperature. Furthermore, the peptides within these hydrolysates lost their ability to trigger mast cell degranulation, suggesting that most of the peptides formed only retained a single relevant IgE-binding epitope.

Other studies have corroborated these findings, indicating that applying high pressure during enzymatic hydrolysis can effectively reduce the antigenicity and IgE-binding attributes of milk protein hydrolysates. This reduction in allergenic potential can be attributed to the increased accessibility of potentially immunogenic hydrophobic regions to enzymes, leading to improved hydrolysis. Consequently, utilizing high pressure during the hydrolysis of milk proteins could be a promising strategy for producing hypoallergenic whey hydrolysates (Bu et al, 2013).

3.4.1.4 Microwave

As reported by El Mecherfi et al., (2019), the simultaneous use of microwave heating and enzymatic proteolysis has demonstrated the ability to enhance the susceptibility of resistant proteins like β-LG to pepsinolysis. This innovative approach offers a novel means to enhance the bioactive characteristics of milk proteins and could be easily incorporated into industrial operations. Furthermore, the extensively hydrolysed whey protein hydrolysates generated using microwave-assisted methods did not trigger allergic reactions, unlike those produced through traditional heating methods.

From an industrial perspective, employing microwave processing for liquid food products substantially enhances system flexibility compared to conventional heating methods, thanks to low inertia and potential energy savings.

3.4.1.5 Ultrasound

In the study of Wang et al., (2020), they investigated the utilization of ultrasound to develop hypoallergenic fresh milk. They assessed how ultrasound affected nature of fresh milk, with a particular focus on colloidal caseins as the basis for their analysis. While the visual appearance of the fresh milk remained mostly unchanged, the application of ultrasound notably reduced its allergenic characteristics. In particular, the efficiency of ultrasound treatment was more pronounced at lower concentrations. Consequently, the researchers expanded their ultrasound approach to diluted fresh milk. Through the dilution of fresh milk with phosphate-buffered saline at various ratios (1:3, 1:10, 1:20, and 1:50 V/V) and subjecting it to ultrasound

for 60 minutes in the presence of 1% Tween 80, they observed a significant increase in milk transparency, despite a persistent cloudy appearance.

Furthermore, the study investigated the effects on IgE-binding capacity and degranulation potential. In alignment with their findings regarding colloidal caseins, the application of ultrasound markedly decreased the fresh milk's ability to bind with IgE. The addition of 1% Tween 80 further enhanced this effect. Additionally, both ultrasound and Tween 80 compromised the fresh milk's capacity to induce degranulation in LAD2 cells. These results conclusively demonstrated that the implementation of ultrasound led to the creation of hypoallergenic fresh milk.

3.4.1.6 Ohmic heating

In the study by Pereira et al., (2020), the immunoreactivity of β-lactoglobulin is closely associated with changes in its structure involving denaturation and aggregation pathways. Recent studies have demonstrated that various types of thermal protein processing can lead to distinct impacts on immunoreactivity. However, the effects of thermal and electrical aspects of ohmic heating (OH) on protein immunoreactivity have not been explored until now. OH, induces changes in protein conformation due to differential exposure of hydrophobic regions, which seems to have a significant role in activating immune system cells.

Exposure to a temperature of 72.5 °C for 15 seconds, regardless of the OH or conventional (COV) treatment type, resulted in a reduction of β-LG by 25-35%. Conversely, heating β-LG at 90 °C for 1 second led to a general increase in immunoreactivity. Interestingly, treatments FOH90_4_25 kHz, which was an ohmic heating with 4 V/cm electric field on 25 kHz and the pasteurization binomial was 90 °C per 1 seconds and FOH90_15_50 Hz –which was also an ohmic heating with the same parameters as mentioned, just on 50 Hz and with 15 V/cm electric field – previously demonstrated immunoreactivity levels comparable to the control (U), indicating that OH treatments, particularly these with rapid heating kinetics and specific electrical conditions, hold the potential to preserve β-LG immunoreactivity.

1. Table β-LG immunoreactivity evaluated through ELISA (Pereira et al., 2020)

Treatment code	Meaning				ng/mL
	Pasteurization Binomial	Treatment	Electric Field (V/cm)	Frequency (Hz)	
U		Untreated (control)			35.7 ± 1.7
OH65_4_25 kHz	65 °C/30 min	Ohmic	4	25 000	30.4 ± 0.8
FOH90_4_25 kHz	90 °C/1 s	Ohmic	4	25 000	35.1 ± 1.8
FOH90_15_50 Hz	90 °C/1 s	Ohmic	15	50	35.2 ± 0.9

The study also investigated the impact of various factors, including thermal and non-thermal variables, on the immunoreactivity of β-LG. The results showed that these variables affected β-LG immunoreactivity through two distinct mechanisms: one involving an increase in the reactivity of the β-LG monomeric form (as observed in the case of OH65_4_25 kHz treatment), and the other involving the alteration of β-LG aggregation (as seen in the case of FOH90_4_25 kHz treatment). However, the relationship between different structural form of β-LG (aggregated vs. monomeric) and their allergenic potential need further exploration.

Correlations between different measurements were observed: increased intrinsic /extrinsic fluorescence and protein unfolding were positively correlated with each other and negatively correlated with circular dichroism (CD) and native-PAGE results. ELISA outcomes negatively correlated with immunoblotting results for the monomeric form of β-LG, indicating that the immunoreactivity of native β-LG might have been underestimated through ELISA. Overall, increased immunoreactivity was linked to denaturation and aggregation of β-LG, as well as changes in fluorescence and CD measurements. Monomeric β-LG's immunoreactivity correlated with structural integrity and higher concentrations of native protein. The study suggested that the certain treatments, such as FOH90_4_25 kHz, factored an intermediate denaturation state of β-LG with reduced aggregates and native structure.

3.4.1.7 Radiofrequency heating

Radiofrequency (RF) heating stand as an emerging alternative technology to traditional thermal methods, finding application in food pasteurization due to its rapid and volumetric heating capabilities. However, concerns have arisen regarding uneven heating patterns. Various studies have investigated RF pasteurization in different liquid food contexts. A comprehensive review by Soto-Reyes et al., (2022), outlines the mechanism of RF heating and the equipment utilized for pasteurizing liquid foods with the aim of controlling harmful microorganisms or spoilage agents.

Creating an efficient RF pasteurization process for liquid foods requires a deep understanding of the dielectric properties of the food in question. These properties influence heating uniformity, temperature dispersion, and heating rate. Continuous RF heating systems' effectiveness may rely on factors such as fluid flow rate, residence time, and absorbed power. Through RF heating, liquid food can be pasteurized, leading to a reduction in microbiological populations while preserving the nutritional and physicochemical integrity of the product.

In the study presented by Zhu et al., (2014), they employed radio-frequency heating and microwave heating for the pasteurization of both cow's and goat's milk, along with an investigation into the dielectric properties of this milk types. The dielectric constants and dielectric loss factors of these milks were determined across a wide frequency range (10-4500 MHz) and temperature range (25-75 °C). Mathematical models were developed to describe the relationship between permittivity's and temperature, and the power penetration depth was explored.

The findings revealed that the dielectric constants of both cow's milk and goat's milk decreased as frequency increased, while the dielectric loss factor decreased at frequencies below 1000 MHz and increased beyond that point. Temperature had an impact on these properties as well, with dielectric constants decreasing with rising temperature at given frequency. For raw cow's milk, the loss factor generally increased with temperature up to around 800 MHz and then decreased thereafter.

Furthermore, the penetration depth decreased with increasing frequency and was less influenced by temperature. This study suggests that radio-frequency heating at 27.12 and 40.68 MHz, as well as microwave heating at 916 MHz, are suitable options for cow's milk pasteurization.

3.4.2 Chemical treatments

3.4.2.1 Acidic treatments – Lactic acid fermentation

Proteolytic enzymes produced by lactic acid bacteria (LAB) during fermentation have complex systems comprising proteinases, peptidases, and transport systems. Such fermentation of milk proteins by Lactobacillus strains can impact milk digestibility and generate bioactive peptides. This proteolysis process can break down epitopes, potentially reducing milk allergenicity. Probiotics, including LAB, have been shown to stimulate the immune system and mitigate immune-mediated diseases. Consumption of fermented products like yoghurt could alleviate atopic symptoms and potentially regulate allergies.

Studies have demonstrated that Lactobacillus fermentation leads to the degradation of milk allergens. Various LAB strains exhibit proteolytic activities towards milk proteins, reducing their allergenicity. Specific LAB strains, such as *Lactobacillus delbrueckii subsp. bulgaricus* CRL 656 and *Lactobacillus fermentum* IFO3956, can reduce IgE recognition of β-LG, α_{s1}-casein, and β-casein through their proteolytic activity.

The degree of change in milk protein antigenicity and allergenicity depends on the LAB species and fermentation conditions. Lactic acid fermentation can significantly decrease the antigenicity of milk proteins, with some studies reporting over 90% reduction. However, this reduction does not necessarily eliminate allergenicity, as some allergenic responses might still persist despite the decrease in antigenicity. The reduction in antigenicity is attributed to the hydrolysis of epitopes by proteolytic enzymes from Lactobacillus strains during fermentation. The variation in antigenicity reduction across studies can be attributed to differences in the hydrolytic ability and specificity of proteinases produced by various LAB strains. These findings offer valuable insights into developing new fermented milk products with diminished antigenic properties (Bu et al., 2013).

3.4.2.2 Enzymatic hydrolysis

In the research conducted by Liu and colleagues in 2012, it was determined that the use of papain in enzymatic hydrolysis is effective in significantly reducing the antigenicity of both α-casein and β-casein. However, complete elimination of casein's antigenicity wasn't achieved through this method. Among the various factors explored, the ratio of enzyme to substrate exhibited the most substantial impact in diminishing the allergenic properties of casein. The pH was also found to exert some influence on reducing the antigenicity of α-casein and β-casein, although to a lesser degree. Response Surface Methodology (RSM) proved to be suitable for concurrently optimizing several independent variables involved in the hydrolysis process. This resulted in the creation of casein hydrolysate with minimal antigenicity for both α-casein and β-casein. The model established was effective in representing the actual correlation between the responses and the independent variables. Notably, the inhibition of anti-α-casein IgG binding and anti-β-casein IgG binding exhibited significant and negative associations with the degree of hydrolysis (DH).

Proteolysis presents an effective method to eliminate allergenic epitopes (Heyman, 1999). To mitigate their allergenic properties, proteins can be enzymatically broken down into small peptides and amino acids. Proteolytic enzymes are found widely across animal, plant, and microbial sources. Some food-grade proteinases have been employed to produce whey protein hydrolysates with reduced allergenicity.

During hydrolysis, variations in enzyme type, hydrolysis conditions, and extent of hydrolysis can lead to differences in peptide composition, residual allergenicity, and taste. Pahud et al., (1985) showed that whey protein antigenicity can be diminished by trypsin hydrolysis. Nakamura et al., (1993) demonstrated that using combinations of enzymes like papain, neutrase, alcalase, and protease S can be more effective in lowering the allergenicity of whey protein compared to single enzymes. Another study indicated that a two-step hydrolysis process using alcalase and papain was most successful in reducing the immunoreactivity of cow's milk whey protein, although some allergenic epitopes remained. The addition of papain to whey protein concentrates hydrolysates prepared with alcalase also improved sensory qualities by reducing bitterness. Additionally, the antigenicity of whey protein concentrate hydrolysates with alcalase can be effectively decreased by optimizing hydrolysis conditions.

Enzymatic protein digestion can generate new antigenic substances. For instance, Haddad et al., (1979) detected serum IgE from allergic patients using total tryptic hydrolysate of β-

lactoglobulin even when no IgE response was observed with the native form. Enzymatic proteolysis may also increase protein allergenicity by exposing more antigenic sites during hydrolysis. Peptides formed by hydrolysis can retain allergenicity if epitopes are present, regardless of their low molecular masses. The choice of enzyme, rather than the extent of hydrolysis or molecular mass distribution of hydrolysates, plays a role in determining residual allergenicity.

Combining enzymatic hydrolysis with prior heat treatment can enhance the hydrolysis of major milk proteins, resulting in reduced allergenicity, Microwave treatment combined with enzymatic hydrolysis has also been explored, showing enhance hydrolysis rates and reduced immunoreactivity.

Whey proteins and peptides derived from their enzymatic proteolysis can influence immune functions such as lymphocyte activation, cytokine secretion and antibody production. Studies indicate that enzymatic hydrolysis can partly reduce the allergenicity of certain proteins.

Hydrolysate formulas have been developed to decrease the allergenicity of cow's milk proteins. Partially and extensively hydrolysed formulas are available, with varying degrees of hydrolysis to address cow's milk allergies. Extensively hydrolysed casein-based formulas are recommended for children with IgE-mediated cow's milk allergy, while partially hydrolysed formulas might be useful for preventing cow's milk allergies in high-risk infants (Bu et al., 2013).

3.5 Hydrolysis

3.5.1 Effects of pH, temperature, enzyme-to-substrate ratio and reaction time

In the experiment by Liu et al., (2012), the influence of pH, temperature, enzyme-to-substrate ratio and reaction time were investigated on the antigenicity of casein hydrolysates prepared by papain. Their research used sensation studies and enzyme-linked immunosorbent assay (ELISA) using α-casein (C-6780; purity 90%), β-casein (C-6905; purity 95%) from Sigma Chemical Company (St. Louis, MO, USA). Commercial casein obtained from Beijing Bio-technology Company (Beijing, China) was used as hydrolysis reaction substrate. Papain used for the hydrolysis of casein was purchased from FangShan Enzyme Preparation Factory (Beijing, China).

To evaluate of the models concerning the antigenicity of casein hydrolysates across four independent variables regression analysis was used. The significance and the fitness of the model were assessed by ANOVA, in addition, the effects of individual terms and their interactions on the responses were evaluated also by ANOVA. The full model revealed that several terms were not significant. The nonsignificant terms were eliminated to fit the full second-order model. For α-casein, the linear effects of temperature and reaction time are not significant. However, the quadratic effects of temperature and reaction time are significant. So, the linear terms should be retained in the model. In addition, β-casein, the linear effect of temperature is not significant, but the interaction effects between temperature and pH is significant. So, the linear term of temperature should be retained in the model. This procedure resulted in the second-order model for α-casein with eight regression terms and that for β-casein with eight regression terms for the fitted model. The P-values for the fitted model showed for α-casein and for β-casein 0.0001, respectively, the adjusted R^2 of the fitted model for α-casein is 0.861 and for β-casein is 0.816. The small P-values and high adjusted R^2 values demonstrate that the model could give a good description of the relationship between responses and independent variables.

Among the studied parameters, the enzyme-to substrate ratio had the most influence on the inhibition of α-casein and β-casein. Furthermore, the negative correlation between the anti-α-casein IgG binding inhibition and the anti-β-casein IgG binding inhibition with the degree of hydrolysis was highly significant.

4 Materials and methods

4.1 Materials

4.1.1 Cow's milk

Different cow's milk was used for the measurements. The following table gives an illustrative indication of the fat content and heat-treated milk used in each measurement.

2. Table The type of milk used in the measurements

Measurement	Heat-treatment	Fat-content
3P design	UHT	skimmed
2P design	Homogenization + Pasteurization	0.1% and 1.4%
Water bath, Ohmic heating, Radiofrequency	UHT	whole

4.1.2 Papain enzyme

For the preparation of enzyme solutions, I used lyophilized protease (not immobilized) produced by HiMedia (HiMedia Laboratories GmbH Marie-Curie-Str.3 64683, Einhausen, Germany).

4.2 Methods

4.2.1 Enzyme solution preparation

To prepare the enzyme solution, a mother solution was made to rehydrate the lyophilized cysteine protease enzyme, to which 0.09 g of papain was weighted on an analytical scale, and then added 10 mL of distilled and sterilized water. During the work, an aseptic working condition was aimed to avoid cross-contamination. In the experiments, different concentrations of the enzyme were used, the required amounts were measured with using an automatic pipette and added it to the cow's milk. The concentrations used in each experiment are indicated in the results section.

4.2.2 Enzymatic hydrolysis of milk protein

Enzymatic hydrolysis was carried out in the following steps:

1. Measuring the quantity of milk to be hydrolysed

For the 3P and 2P modelling, 150 mL of milk was used for hydrolysis, while 100 mL of milk was used for the comparison of the different heating methods.

2. Pre-incubation for 1 hour

To reduce the allergenicity level more effectively, a one-hour preincubation was used to allow the conformational epitopes to open up during the heat treatment. Preincubation was carried out at 50 °C for all the three measurements.

3. Adding papain enzyme to cow's milk

For the 3P model, I added the enzyme to the milk at 3 different concentrations: 0.008, 0.012, 0.016 g/L. Subsequently, the optimized concentration of 0.012 g/L was used for the further measurements. The papain enzyme was added to the preincubated cow's milk using an automatic pipette as indicated above.

4. Enzymatic hydrolysis

The hydrolysis temperature ranged between 40-60 °C and the hydrolysis time between 10-20 min with different combinations during the optimization. Optimized hydrolysis temperature

and time was used for the three different heating methods. Enzymatic hydrolysis time and temperature are indicated in the results section for each type of measurement.

5. Inhibition for 30 minutes

Inhibition lasted 30 minutes for each measurement, which was carried out at 70 °C to stop the enzyme reaction.

6. Sample selecting

After inhibition, take samples for further measurements.

4.2.3 Allergenicity by ELISA method

The most promising methods for the analytical determination of the immunocomplex formed are marker enzymes. The marker enzymes can be linked to antibodies or antigens so that the complex has both immunological and enzyme activity.

This new enzyme immunoassay is variously referred to in the literature as according to a certain degree of variation in implementation. It is commonly referred to as EIA, which is short for Enzyme ImmunoAssay, and ELISA, which is a heterogenous technique that stands for Enzyme-Linked ImmunoSorbent Assay (Hegedűs, Gy., 2003).

4.2.3.1 The principle of the ELISA method

Most EIA techniques include at least one separation step, in which the given free target compound and its conjugate (hapten-enzyme or hapten-protein conjugate) from fractions not bound to the antibody are separated. This allows the activity of the bound (or free) enzyme to be measured. Enzyme activity is usually measured by a colour reaction with the chromophore substrate of the marker enzyme. This allows the amount or concentration of free antigen bound to the antibody (the compound to be detected) to be determined the sample.

The complex stability of the antigen-antibody formed in the reaction depends on the space of the two components. The reaction continuous until equilibrium is reached, at which point some of the reagents are bound and some are free. The equilibrium state can be described by the law of mass action:

$$Ag + Ab \rightleftharpoons [Ag.Ab] \tag{4.1}$$

where,

- [Ag]: Free antigen concentration
- [Ab]: Free antibody concentration
- [Ag.Ab]: Concentration of the antigen-antibody complex

On this basis, it can be concluded that for a given amount of antibody, the equilibrium the distribution of antigen (bounded/free) depends on the total amount of antigen present. The ELISA technique, the antibody (or the hydrogen) is coupled to a marker enzyme and the colorimetric substrate of this enzyme is used for detection.

The labelled (Ag*) and unlabelled antigen (Ag) present in the ELISA system are competitively linked to the antibodies. The reaction is continued until equilibrium is reached.

$$Ag + Ag^* + Ab \rightleftharpoons [Ag.Ab] + [Ag^*.Ab] \tag{4.2}$$

For a given amount of antibody and labelled antigen, the ratio of the bounded to free fraction of the non is determined by the amount of unbound antigen (Hegedűs, Gy., 2003).

4.2.3.2 ELISA kit

Percentage allergenicity reduction levels were tested using the AgraQuant® milk allergen ELISA kit from RomerLabs®. The test was performed according to the measurement instruction provided by the manufacturer. The absorbance of each sample was then measured with a spectrometer at 450 nm, the wavelength recommended by the company.

7. Figure The ELISA kit

4.2.4 Determination of protein concentration

The enzymatic hydrolysis reaction's ideal operating conditions were established using the „3P" approach. To create a factorial design and response surface plot, the Statistica 11 software was employed.

4.2.5 Anti-ACE activity

Recombinant angiotensin converting enzyme was received from Division of Clinical Physiology, Institute of Cardiology, University of Debrecen, Hungary. The substrate Abz-FRK(Dnp)-P, the recombinant angiotensin-converting enzyme, and supernatants of the milk samples were used to determine the IC 50 values of samples. Enzymatic reaction mixture (final volume 200 µL in each well), consisted of 100 mM of TRIS HCl (pH 7), 50 mM of sodium chloride, 10 µM of zinc chloride, 15 µM of substrate Abz-FRK(Dnp)-P (Sigma-Aldrich, Schnelldorf, Germany), recombinant angiotensin converting enzyme (amount of the recombinant angiotensin converting enzyme was chosen to result in about 10-fold activity than that in human serum), and supernatants of milks and corresponding fermentation broth (in a dilution range of 10-fold to 10 6 -fold) was used to determine IC 50 value of samples. Activity of angiotensin-converting enzyme in the absence of the milk samples was used to define uninhibited activity. Reaction was initiated by the addition of substrate. The level of inhibition

was calculated as % of uninhibited activity in each plate. Measurements were performed in a fluorescent plate reader (BMG Novostar, BMG Labtech, Germany) at temperature 37 o C. Changes in optical density were measured with excitation wavelength of 340 nm and emission wavelength of 405 nm for at least 20 cycles (Nath et al., 2021).

4.2.6 Antioxidant capacity

4.2.6.1 Ferric Reducing Ability of Plasma (FRAP) Assay

2,4,6-Tris(2-pyridyl)-S-triazine was procured from the Sigma-Aldrich (Sigma-Aldrich, Schnelldorf, Germany). Appropriate diluted 100 µL of supernatant was mixed with 2.9 mL of reagent (5 mL of 20 mM of ferric chloride + 5 mL of 10 mM of 2,4,6-Tris(2-pyridyl)-s-triazine with 40 mM of hydrochloric acid + 50 mL of 300 mM of sodium acetate buffer, pH 3.6) and incubated at temperature 35 °C for 30 min in an incubator (HACH, Düsseldorf, Germany). In this method, reduction of the Fe^{3+} - TPTZ (ferrictripyridyltriazine) complex to the Fe 2+ -TPTZ at low pH in the presence of antioxidant peptides/ proteins produced blue colour, which was measured with wavelength 593 nm in a UV-Vis spectrophotometer (EvolutionTM 300; Thermo ScientificTM, Waltham MA, USA) in RT. Ascorbic acid (99.7%, Merck, Darmstadt, Germany) was considered as a reference of antioxidant capacity of milk samples. A blank sample was prepared with 100 µL of DI water and 2.9 mL of reagent for spectrophotometric analysis (Nath et al., 2021).

4.2.6.2 2,2-Diphenyl-1-picryldrazyl (DDPH) Radical- Scavenging Assay

2,2-Diphenyl-1-Picrylhydrazyl (DDPH) was purchased from the Sigma-Aldrich (Sigma-Aldrich, Schnelldorf, Germany). Assay was performed with 100 µL of supernatant and 3.9 mL of 6×10^{-5} M of DDPH-methanol solution. The mixtures were measured at 517 nm with a UV-Vis spectrophotometer (Evolution™ 300; Thermo Scientific™, Waltham MA, USA). DDPH – radical scavenging activity was calculated based on percentage change of absorbance from control (Nath et al., 2021).

4.2.7 Determination of Protein Concentration

Appropriately diluted 100 µL of supernatant and 3 mL of Bradford reagent (Sigma-Aldrich, Schnelldorf, Germany) were mixed and vortexed. Subsequently, mixture was incubated at RT for 30 minutes in an incubator (HACH, Düsseldorf, Germany). Spectrophotometric analysis was performed with wavelength 580 nm in a UV-Vis spectrophotometer (Evolution™ 300; Thermo Scientific™, Waltham, MA, USA) at RT. Bovine serum albumin (Sigma-Aldrich, Schnelldorf, Germany) was used as a standard assay. During colorimetric determination, a blank sample, prepared with 100 µL of DI water and 3 mL of Bradford reagent was used (Nath et al., 2021).

4.2.8 Electrophoresis, SDS-page

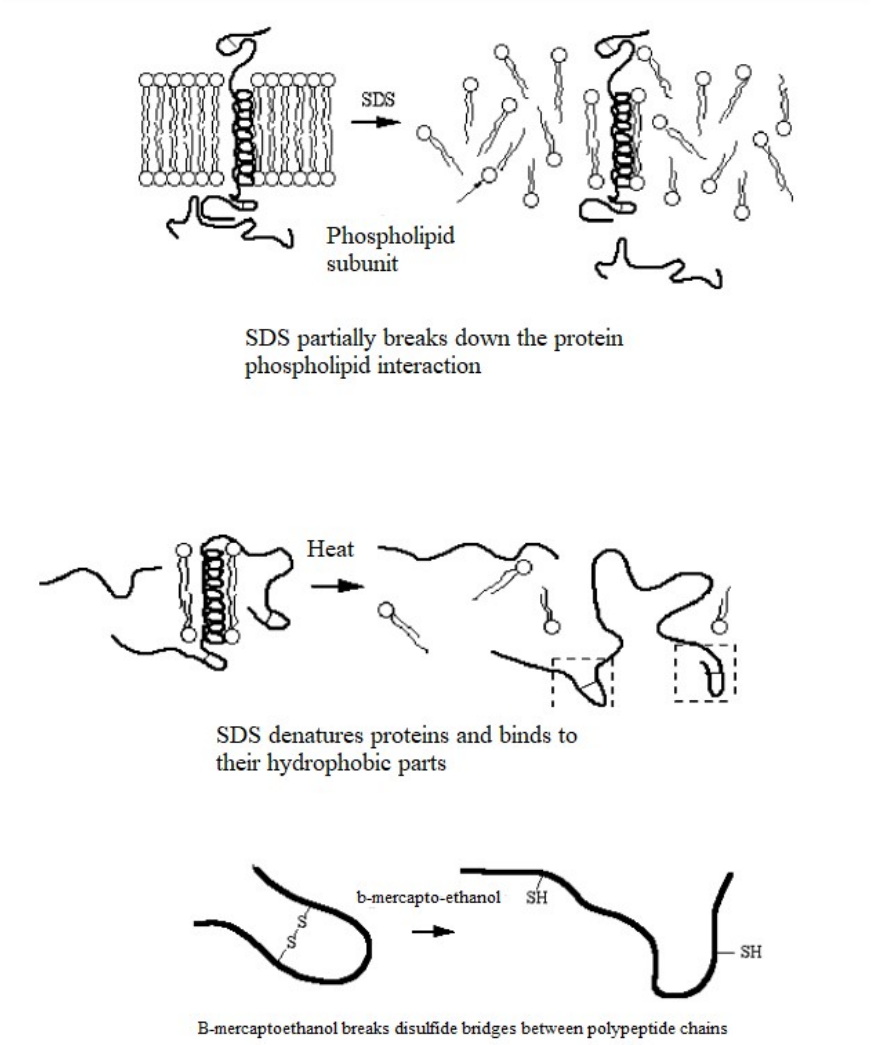
At neutral pH, proteins are negatively or positively charged depending on their primary structure positively. Macromolecules in solution are electrically charged by an electric field, size and shape. This process is called electrophoresis.

In SDS-PAGE (Sodium Dodecyl Sulphate- PolyAcrylamide Gel Electrophoresis), the migration takes place in a highly hydrated polyacrylamide gel with adjustable pore diameter. The solvent used to precipitate the proteins contains an anionic detergent, sodium dodecyl sulphate, which binds to the hydrophobic part of the proteins when exposed to heat (60-100 °C), promotes their unpacking and, being anionic detergent, it imparts a negative charge. By adding a reducing agent to the protein solution (β-mercaptoethanol), the disulfide bridges (-S-S-) between the polypeptide chains are broken. Acrylamide can polymerize in aqueous solution the presence of suitable catalyst (ammonium peroxydyl sulphate, APS) and initiator (tetramethylene ethylenediamine, TEMED, and during the reaction, a high molecular weight linear polymer, so-called polyacrylamide is formed.

The spontaneous decomposition of APS leads to the formation of free radical, but this free radical is not able to initiate polymerisation by breaking the double bond of acrylamide, but it is able to excite the TEMED molecule and the resulting free radical able to initiate polymerisation. If a cross-linking agent, N, N-methylene-bisacrylamide, is also used, „bridges” are formed between the long polyacrylamide chains and a cross-linked gel is formed.

During electrophoresis, the proteins are run in this gel. The process has a particularly high resolution. This is because the molecules in the gel separate in size and shape at the same time as the separation based on the difference in relative charges, the gel acts as a molecular sieve. This molecular filtering effect is determined by the average pore size of the gel, which can be varied within a wide range by a suitable choice of the concentration of the acrylamide monomer and the percentage of methylene bis-acrylamide cross-linking agent. The mechanical properties of the gel are favourable in the range of about 4-20% acrylamide concentration.

Polyacrylamide does not contain any charged groups which would adversely affect electrophoresis separation. It is a chemically inert, stable compound, does not interfere with staining reactions for protein detection, and is compatible with most commonly used buffer systems (Kulcsár and Molnár, 2013).



8. Figure Denaturation of proteins (Kulcsár and Molnár, 2013)

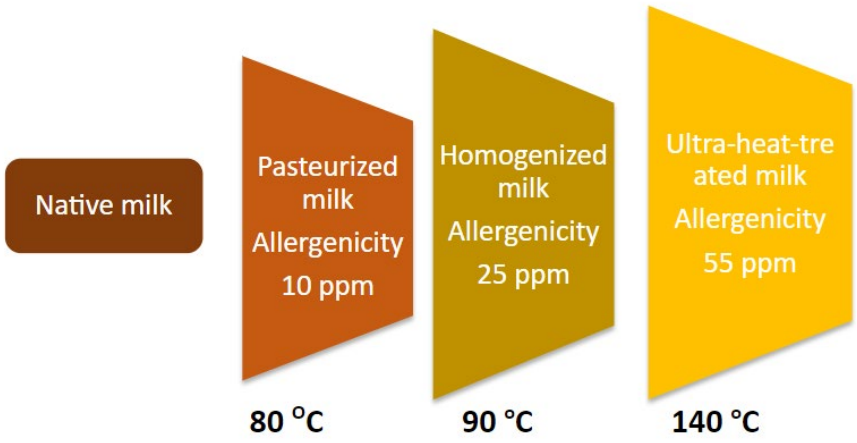
4.2.8.1 SDS-PAGE measurement method

All chemicals required for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were procured from Bio-Rad (Bio-Rad, Hercules, USA). A vertical electrophoresis system (Bio-Rad Mini Protean 3 Cell along with power PAC1000) was used in experiment. Electrophoresis separation of proteins and peptides was performed by constant 200 V, 54 mA and 11 W. Dilution of samples was performed with Laemmli sample buffer (2X) and 10% of 2-mercaptoethanol. In individual well within stacking gel, 10 µL of appropriate diluted sample was loaded and running time was 60 min at RT. For gel staining purpose, 0.2% of Coomassie Brilliant Blue R250 in 9% of acetic acid (96%) – 45% of ethanol was used. Gel staining was performed for 30 min at RT. Subsequently, de-staining of gel was performed with 50% (volume basis) of methanol-water an 10% (volume basis) of acetic acid at RT. Gel Doc System 2000 (Bio-Rad, CA, USA) was used to capture the gel image.

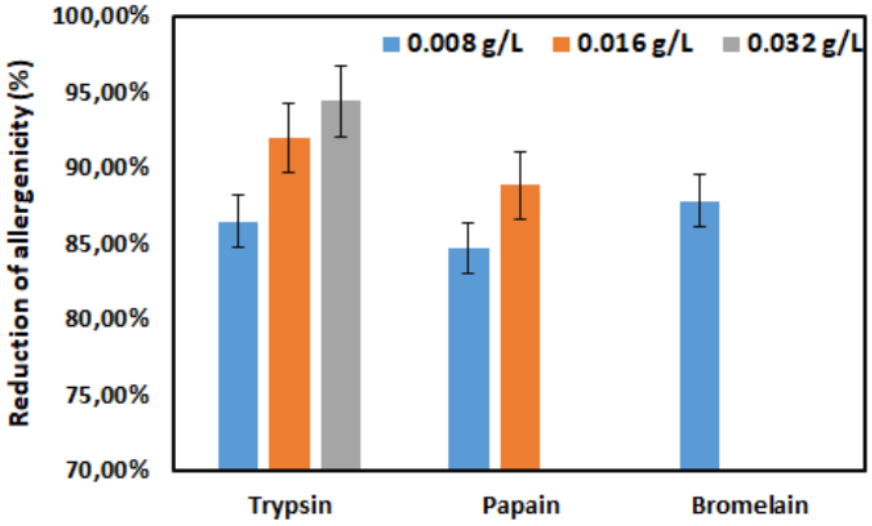
5 Results and discussion

5.1 Allergenicity level during the CM processing

A comparison of the level of allergenicity of cow's milks preserved by different processes can be seen in **Figure 9**. It can be clearly observed that the more the milk is subjected to heat treatment, the more the level of allergenicity increases. The reason for this is that the secondary structure of the protein breaks down under the influence of heat and the bounds between the conformational epitopes are broken, thereby increasing the level of detectable allergenicity. As a conclusion of this result, it can be said that in order to achieve the greatest percentage reduction in the level of allergenicity, the use of the UHT cow's milk is recommended.

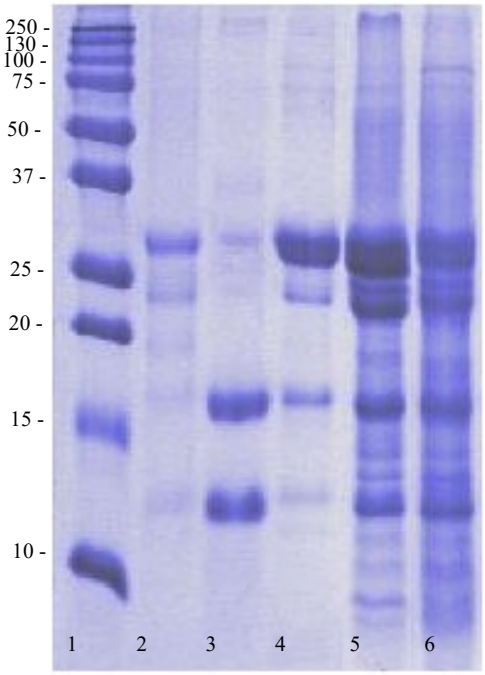


9. Figure Allergenicity during milk processing



10. Figure Reduction of allergenicity with different concentrations of papain's and temperatures in hydrolysis reaction

Figure 10 depicts the percentage reduction in allergenicity of cow's milk achieved when employing varying enzymes at distinct concentrations. With the trypsin enzyme, a more pronounced decrease in allergenicity is observed compared to papain at equivalent concentrations. Despite this, considering the numerous cited benefits of papain, its utilization is recommended over animal-derived enzymes. The impact of bromelain, the pineapple protease enzyme, on allergenicity level also demonstrated a positive outcome.

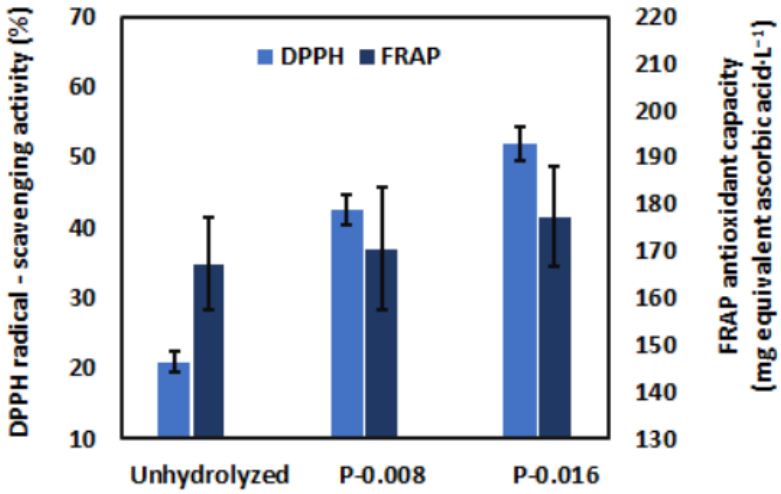


11. Figure SDS-PAGE image of proteins and peptides in unhydrolyzed and papain-hydrolysed milk with the concentration of 0.008 g/L and 0.016 g/L

1. Mw – Marker Proteins (250, 130, 100, 75, 50, 37, 25, 20, 15, 10 kDa)
2. Casein
3. β-LG (~18 kDa), α-LA (~14 kDa)
4. Unhydrolyzed CM
5. P-0.008 – papain enzyme hydrolysed CM with the concentration of 0.008 g/L
6. P-0.016 – papain enzyme hydrolysed CM with the concentration of 0.016 g/L

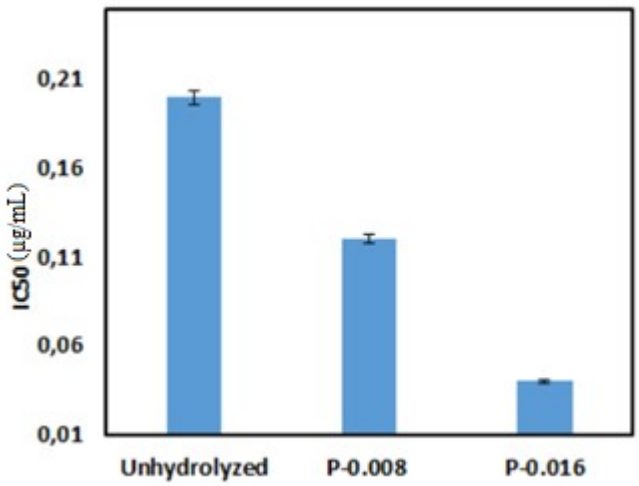
It is evident that the CM sample contains various proteins, including immunoglobulin, lactoferrin, lactoperoxidase, bovine serum albumin, α-casein, κ-casein, β-lactoglobulin, and α-lactalbumin, with a molecular weight of ~150 kDa, ~80 kDa, ~78 kDa, ~66 kDa, ~25 kDa, ~24 kDa, ~20 kDa, ~18 kDa and ~14 kDa. Additionally, there is a protein band at approximately 37 kDa on the SDS-PAGE image. This band may represent a dimer of β-lactoglobulin or a

combination of κ-casein and α-lactalbumin or β-lactoglobulin and α-lactalbumin. The SDS-PAGE image illustrates that the hydrolysis of milk proteins and the formation of lower molecular weight peptides increase gradually due to the enzymatic digestion of the proteins in the milk sample (as observed in **Figure 11** in band 5 and 6).



12. Figure Antioxidant capacity in unhydrolyzed and papain-hydrolysed milk

Figure 12 illustrates the antioxidant capacity assessed through DDPH and FRAD. In terms of the percentage of DDPH radical -scavenging activity, an increase of twofold can be observed between the unhydrolyzed and P-0.008 sample. However, for the sample subjected to hydrolysis with a higher concentration of papain enzyme (P-0.016), such a rapid surge isn't observed; nevertheless, a higher value is still noticeable. Upon investigating the FRAP antioxidant capacity, a minor enhancement becomes apparent with the escalation of enzyme concentration. The milk protein undergoes enzymatic hydrolysis using papain, resulting in peptides that have reduced molecular weight and exhibit functional properties such as antioxidants and anti-ACE effects.

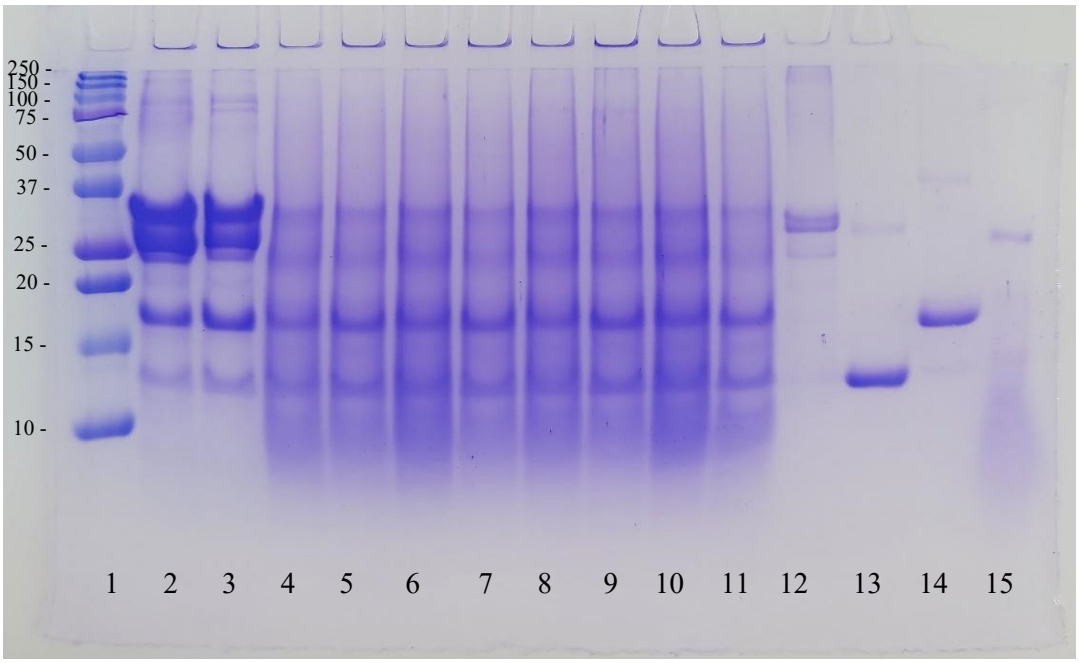


13. Figure Ant-ACE activity in unhydrolyzed and papain-hydrolysed milk

Drawing insights from the outcomes of the Ant-ACE activity assessments, which involved evaluating unhydrolyzed and papain-hydrolysed cow's milk samples at concentrations of 0.008 g/L and 0.016 g/L, it's evident that the unhydrolyzed CM exhibits a significantly greater IC50 value in comparison to the unhydrolyzed. It can be concluded for the P-0.008 sample, there was an approximately ten-fold decrease in IC50 value following papain enzyme hydrolysis. Similarly, doubling the enzyme concentration resulted in a proportional halving of the IC50 level for the P-0.016 sample.

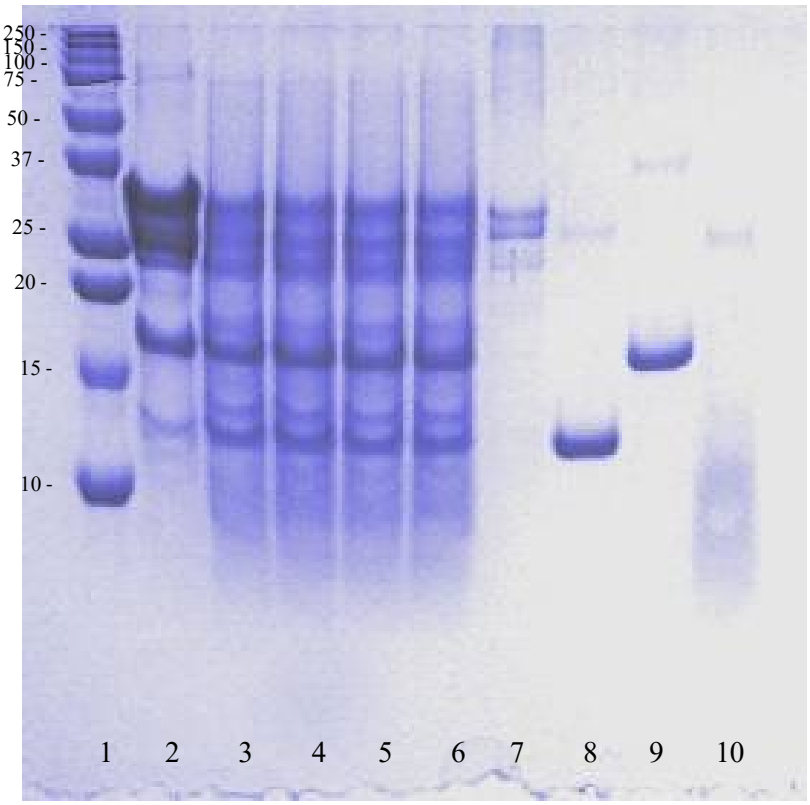
5.2 SDS-PAGE

In the industrial sector, raw milk is placed in a 6 °C cooling tank for 6 or 24 hours before being processed, depending on how busy the production line is, and production breaks caused by work shifts. The following SDS-PAGE tests were performed to investigate the effect of the enzyme papain on cow's milk during this storage. In addition, tests were also performed on UHT and ESL cow's milk to investigate whether the enzyme performs any hydrolysis at this temperature. **Figure 15** shows the experiment with raw milk, while **Figure 14** shows the electrophoresis study with UHT and ESL milk.



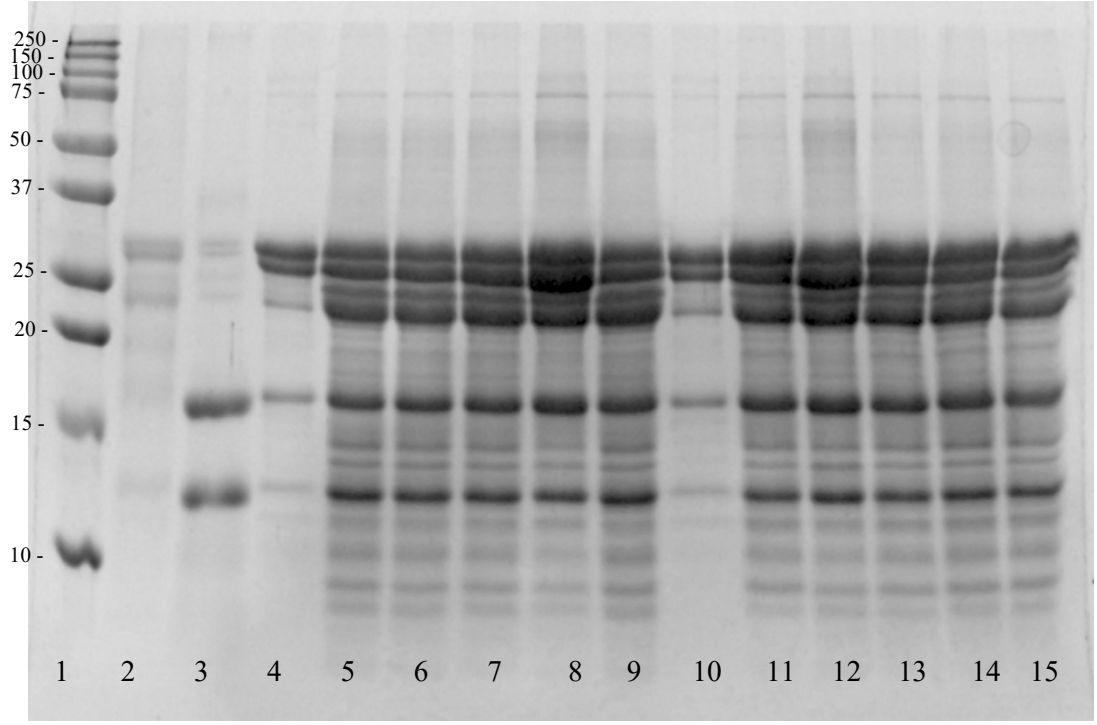
14. Figure SDS-PAGE of proteins and peptides in unhydrolyzed and papain hydrolysed UHT and ESL milk

1. Mw (250 ,150, 100, 75, 50, 37, 25, 20, 15, 10 kDa)
2. New UHT
3. New ESL
4. UHT 6 hours incubation at 6 °C, no hydrolysis
5. ESL 6 hours incubation at 6 °C, no hydrolysis
6. UHT 6 hours incubation at 6 °C + hydrolysis
7. ESL 6 hours incubation at 6 °C + hydrolysis
8. UHT 24 hours incubation at 6 °C, no hydrolysis
9. ESL 24 hours incubation at 6 °C, no hydrolysis
10. UHT 24 hours incubation at 6 °C + hydrolysis
11. ESL 24 hours incubation at 6 °C + hydrolysis
12. Casein
13. α-LA
14. β-LG
15. Papain



15. Figure SDS-PAGE of proteins and peptides in unhydrolyzed and papain hydrolysed UHT milk

1. Mw (250 ,150, 100, 75, 50, 37, 25, 20, 15, 10 kDa)
2. NP = no hydrolysis
3. NP6 = sample+enzyme→incubation at 6°C for 6 hours→inhibition at 70°C
4. P6H = sample+enzyme→incubation at 6°C for 6 hours→hydrolysis at 60 °C for 30 min→ inhibition at 70°C
5. NP24 = sample+enzyme→incubation at 6°C for 6 hours→inhibition at 70 °C
6. P24H = sample+enzyme→incubation at 6°C for 6 hours→hydrolysis at 60 °C for 30 min→inhibition at 70°C
7. Casein (Sigma C5890)
8. α-LA
9. β-LG
10. Papain



16. Figure Pasteurized and homogenized CM's hydrolysed at different parameters with two different milk fat contents

1. Mw (250 ,150, 100, 75, 50, 37, 25, 20, 15, 10 kDa)
2. Casein
3. α-LA + β-LG
4. C= control CM 0.1% fat content
5. P6 papain enzyme, hydrolysis at 40 °C, for 10 min, 0.1% fat content
6. P6 papain enzyme, hydrolysis at 40 °C, for 15 min, 0.1% fat content
7. P6 papain enzyme, hydrolysis at 50 °C, for 10 min, 0.1% fat content
8. P6 papain enzyme, hydrolysis at 50 °C, for 15 min, 0.1% fat content
9. P6 papain enzyme, hydrolysis at 60 °C, for 10 min, 0.1% fat content
10. C = control CM 1.4% fat content
11. P6 papain enzyme, hydrolysis at 40 °C, for 10 min, 1.4% fat content
12. P6 papain enzyme, hydrolysis at 40 °C, for 15 min, 1.4% fat content
13. P6 papain enzyme, hydrolysis at 50 °C, for 10 min, 1.4% fat content
14. P6 papain enzyme hydrolysis at 50 °C, for 15 min, 1.4% fat content
15. P6 papain enzyme, hydrolysis at 60 °C, for 10 °C, 1.4% fat content

The control (C) and papain hydrolysed (P) samples are different, with the P samples having more protein of a given molecular weight. No significant difference is seen between the P samples.

For all three SDS-page analyses, it can be concluded that the proteins present in the initial control samples remained detectable even post hydrolysis. However, several proteins with reduced molecular weight emerged following hydrolysis, as observed in individual protein bands.

When comparing the control samples with the hydrolysed ones in **Figure 14** and **Figure 15**, it's evident that proteins initially identified, like casein, α-lactalbumin, β-lactoglobulin, were found in significantly larger amounts prior to EH. In both cases, it can be seen that the casein proteins were present in much higher amounts in unhydrolyzed CM. **Figure 14** does not exhibit any noteworthy distinction between the ESL and UHT milk samples.

In the context of the SDS-PAGE shown in **Figure 16**, where assessments were conducted on pasteurized and homogenized milk samples, it becomes evident that the presence of milk proteins was markedly less detectable in the untreated milk compared to the state after enzymatic hydrolysis (EH). This situation is also notably reflected in the allergenicity level examination detailed in **Table 6**, which displays an elevation in the allergenicity level. This phenomenon arises from the fact that the milk underwent heat treatment at a lower temperature, leading to the unfolding of concealed epitope bonds during the EH, consequently resulting in an escalated level of allergenicity.

5.3 ELISA method

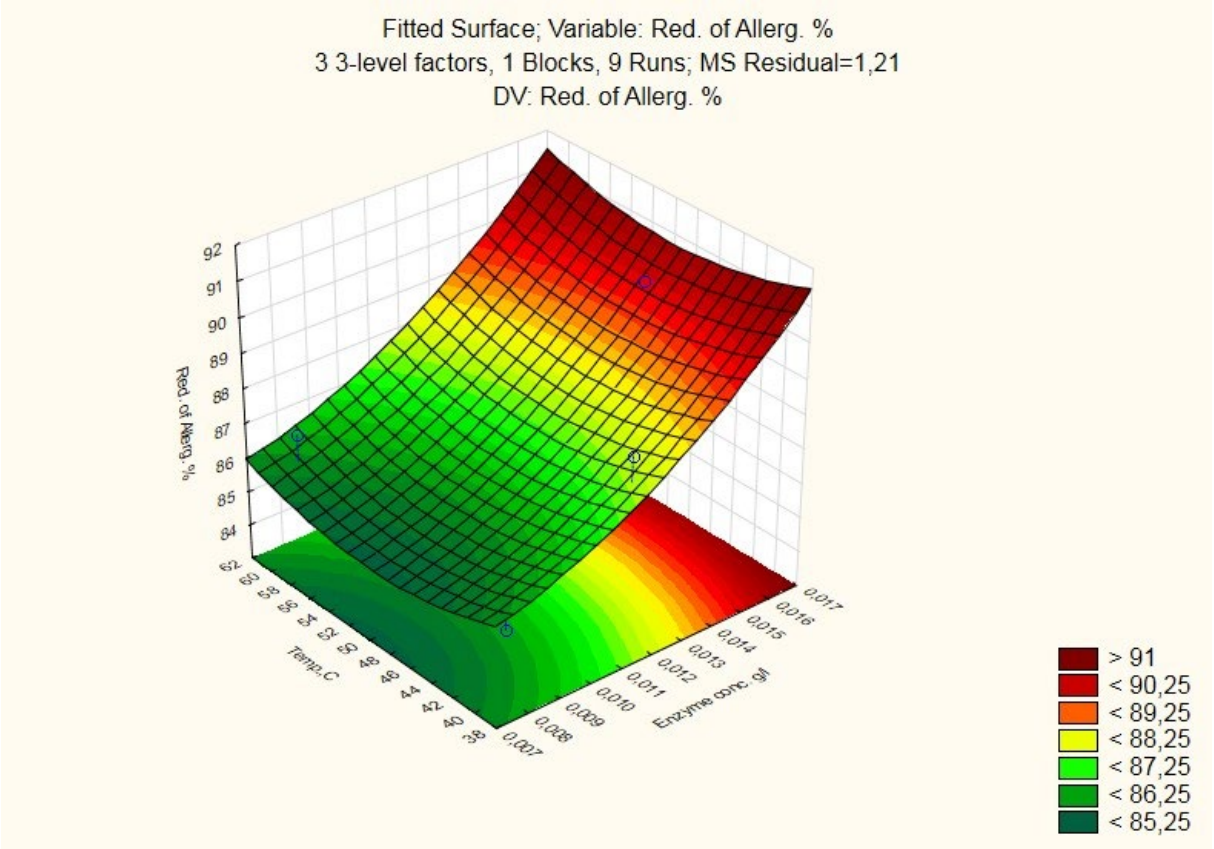
5.3.1 UHT milk (3^P design)

3. Table The allergenicity level (AL) reduction during the enzymatic hydrolysis by different time and temperatures

	Enzyme conc. (g/L)	Temperature (°C)	Time (min)	AL in CM (mg/kg)	AL reduction (%)
UHT Milk - control				60 000	
CM+enzyme sample 1	0.008	40	10	9 000	85
CM+enzyme sample 2	0.008	50	20	58 000	3.3
CM+enzyme sample 3	0.008	60	15	8 000	86.7
CM+enzyme sample 4	0.012	40	20	7 000	88.3

CM+enzyme sample 5	0.012	50	15	8 000	86.7
CM+enzyme sample 6	0.012	60	10	8 000	86.7
CM+enzyme sample 7	0.016	40	15	6 000	90
CM+enzyme sample 8	0.016	50	10	6 000	90
CM+enzyme sample 9	0.016	60	20	6 000	90

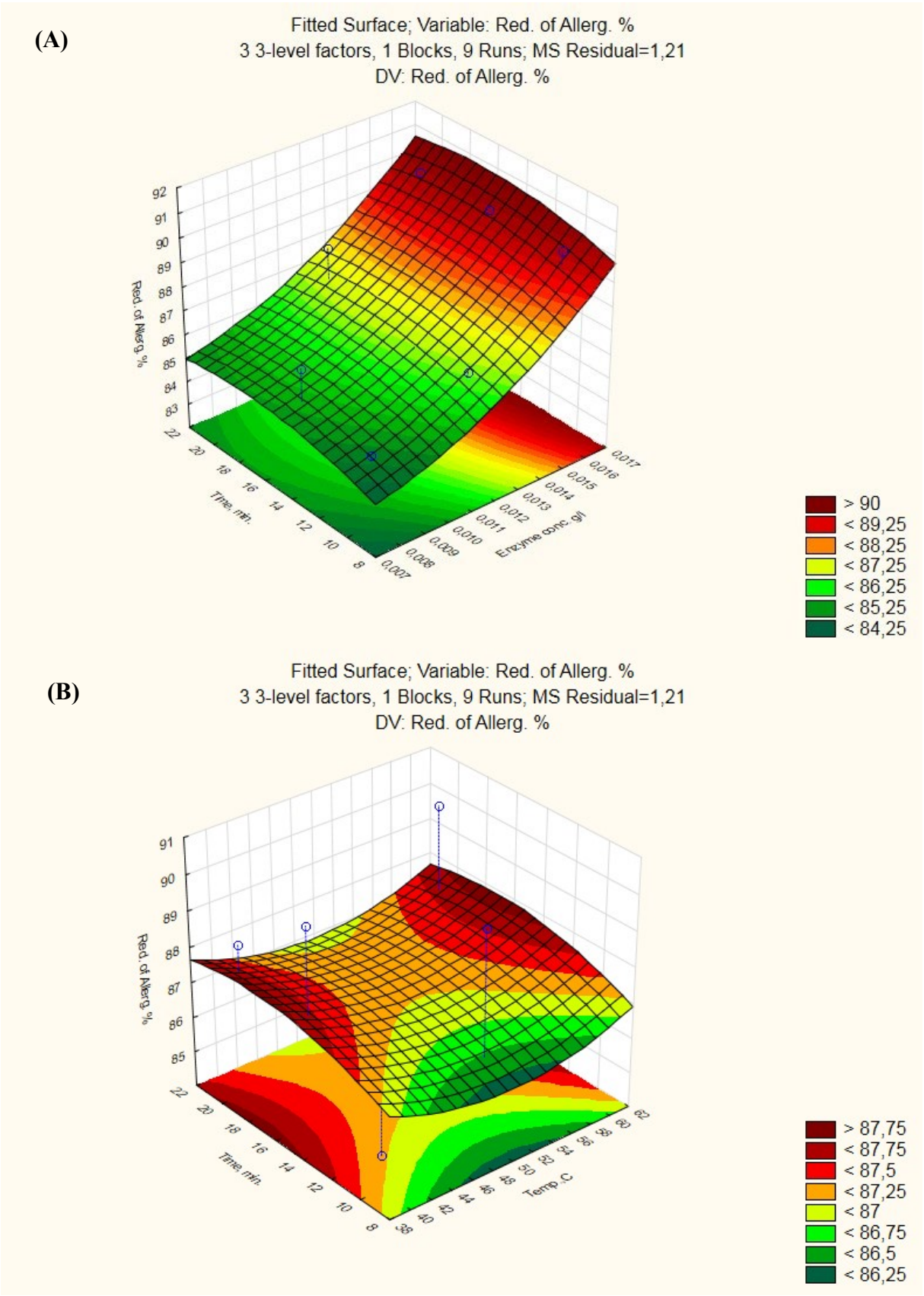
In 3^P modelling, enzyme concentration, hydrolysis temperature and time were modelled. UHT treated milk was used for the experiment, which was performed as described in section 4.2.2. Almost all measurements show a reduction in allergenicity levels above 80%. The greatest reduction in allergenicity was observed for papain enzyme a 0.016 g/L, which presents a 90% reduction in CM. This result is exceptionally good, although using papain at this concentration gave a physical separation and bitter taste. These characteristics were not detectable with the papain enzyme used in a lower concentration; therefore, this amount was used for subsequent measurements.



17. Figure Reduction of the allergen activity level as a function of hydrolysis temperature and enzyme concentration

The change in percentage allergen reduction as a function of temperature and enzyme concentration can be read from the surface diagram in **Figure 17**. It can be observed that a more effective reduction of allergenicity can be achieved with higher enzyme concentration. In addition, the temperature does not have as much influence on the hydrolysis process as the enzyme concentration. It can be concluded that in the case of UHT milk, with the addition of a very small amount of papain and a hydrolysis temperature between 40-60 °C, an allergenicity reduction of 85.25-86.25% can be achieved.

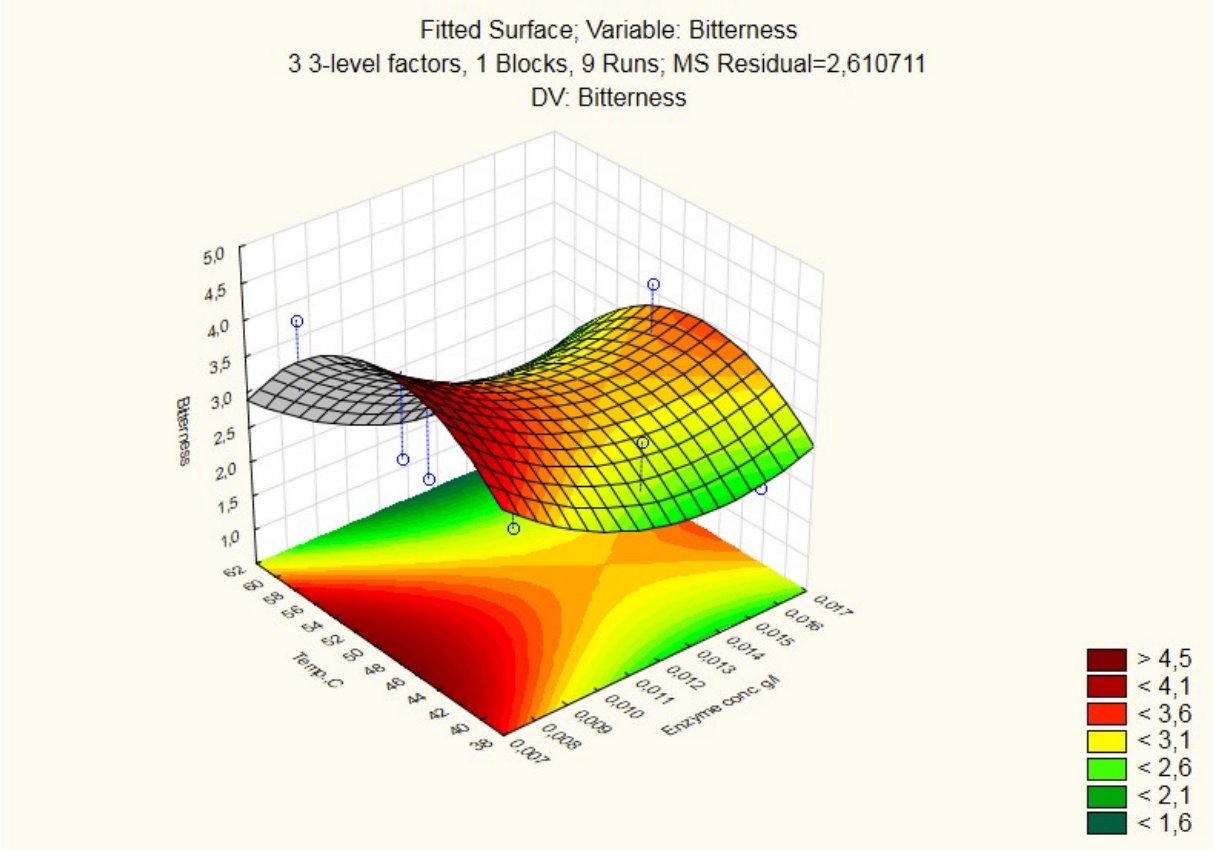
In **Figure 18 (A)**, the percentage reduction in allergenicity is also visible on the vertical axis, but in this case, it is examined as a function of time and enzyme concentration. It can be established here, as in the previous one, that the concentration of the enzyme has a greater effect during the reduction of allergens, while a significant difference is barely visible in the case of time. Nevertheless, it can be determined that a more substantial decrease can be attained by prolonging the EH duration.



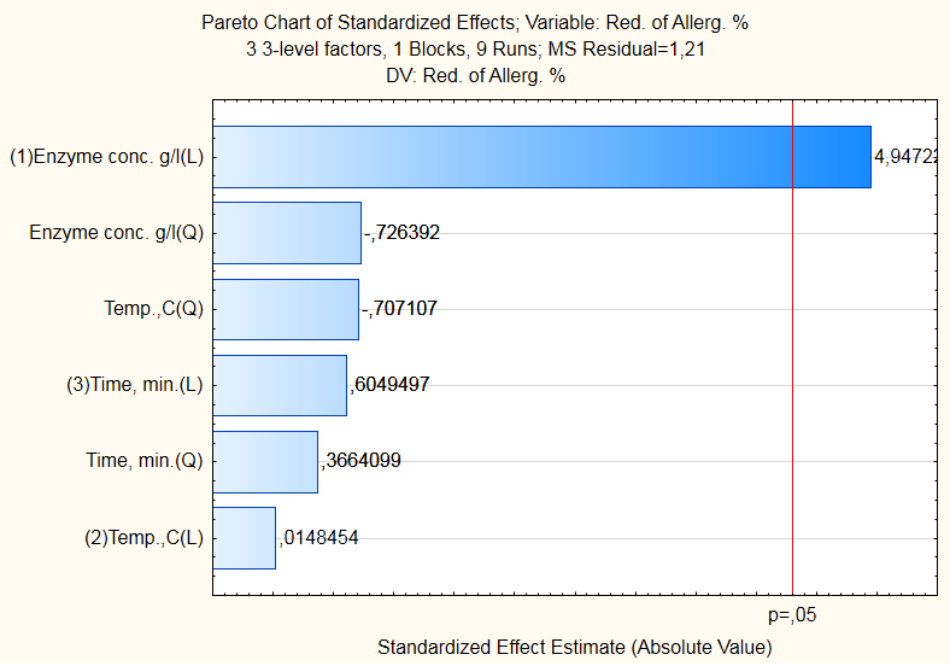
18. Figure Reduction of allergen activity: **(A)** as a function of hydrolysis time and enzyme concentration; **(B)** as a function of hydrolysis time and temperature

Regarding **Figure 18 (B)**, the vertical axis continuous to represent the percentage reduction of allergen content, but now we analyse this in relation to the two less influent variables, specifically the hydrolysis time and temperature. The surface diagram illustrates that the optimal of allergen reduction in CM can be achieved within the range of 14 to 20 minutes of hydrolysis time and a temperature of 56 to 60 °C.

The y-axis of **Figure 19** depicts the evaluation of cow's milk bitterness as influenced by variations in protease enzyme concentration and temperature during the EH. The most pronounced bitter flavour was noted when utilizing a papain concentration of 0.007 g/L and subjecting the enzymatic hydrolysis temperatures between 44 and 52 °C. The lowest levels were detected at 60 °C and within the concentration rang of 0.012 to 0.013 g/L. Similar minimal bitterness was observed at lower temperatures (38-40 °C) along with enzyme concentrations of 0.012 and 0.016 g/L. However, upon referring to **Table 3**, where the concentration of 0.016 g/L showed the highest percentage allergenicity level reduction, the bitter taste sensation was found to be high and thus beneficial for consumption.



19. Figure Dependence of bitter taste on enzyme concentration and hydrolysis temperature



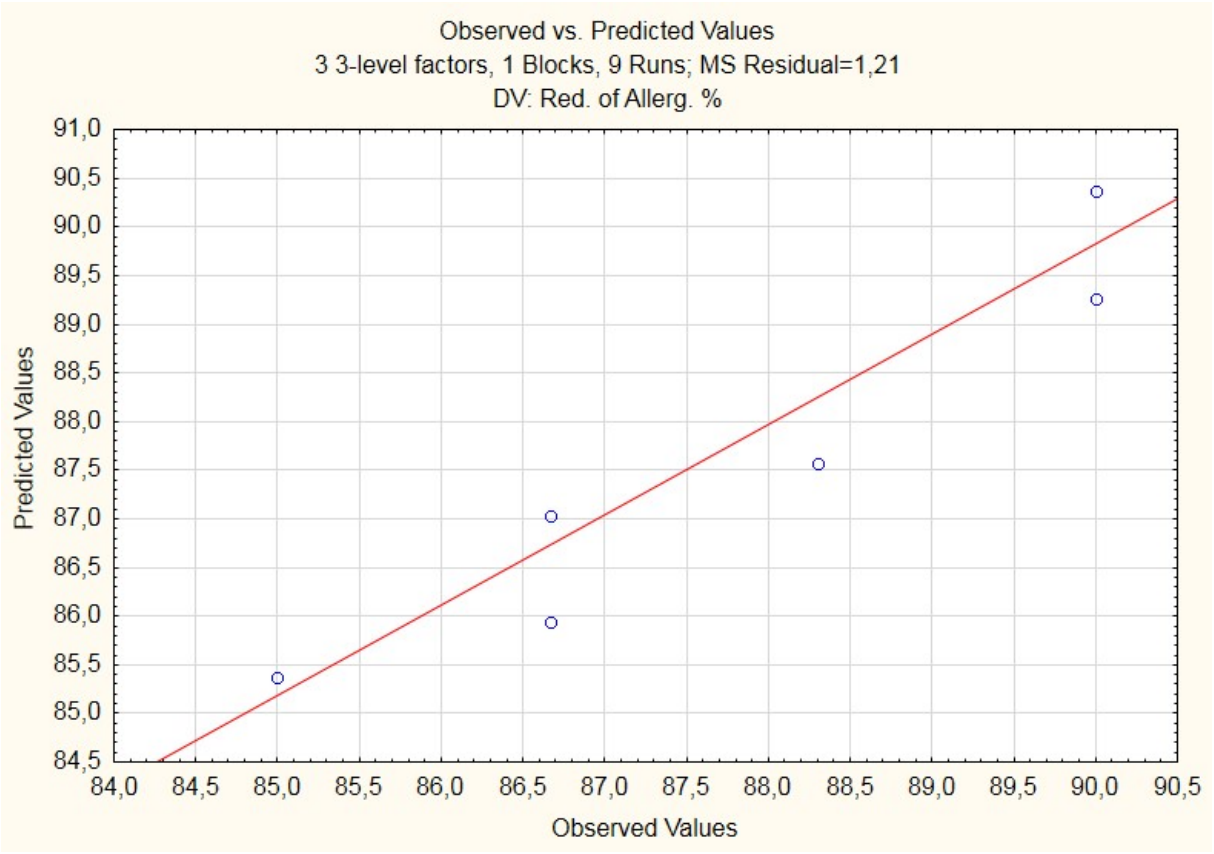
20. Figure Pareto plot of standardized effects for the percentage reduction in allergenic activity and the independent variables (papain concentration, hydrolysis reaction temperature and hydrolysis time)

4. Table ANOVA test for the 3-level factor; Enzyme concentration (g/L), Temperature (°C), and time (minute)

Factor	ANOVA; Var.: Red. of Allerg. %; R-sqr=,92858; Adj:,71432(3**(3-1) fractional design, 1 block, 9 runs, 3-level factors, ; MS Residual=0.21 DV:Red. of Allerg. %				
	SS	df	MS	F	p
(1) Enzyme conc. g/L (L)	29.61482	1	29.61482	24.47506	0,038513
Enzyme conc. g/L (Q)	0.63845	1	0.63845	0.52764	0.543109
(2) Temp., °C (L)	0.00027	1	0.00027	0.00022	0.989503
Temp., °C (Q)	0.60500	1	0.60500	0.50000	0.552786
(3) Time, min (L)	0.44282	1	0.44282	0.36596	0.606708
Time, min (Q)	0,16245	1	0.16245	0.13426	0.749191
Error	2.42000	2	1.21000		
Total SS	33.88380	8			

During the execution of the ANOVA test (**Table 4**), it can be observed, just as with the Pareto diagram (**Figure 20**), that only the effect of enzyme concentration causes significant difference

during the enzymatic hydrolysis, with a 5% significance level, regarding the percentage allergenicity reduction. In the case of the other independent variables, no significant difference can be observed, however, these factors are not negligible in order to create an accurate model. Based on the diagram, it can be concluded that there is a linear relationship between the enzyme concentration and the percentage reduction in allergenicity.



21. Figure Plot of observed and predicted values for the percentage reduction of allergens

The actual measurements closely match the linear trend depicted by the predicted values, suggesting that the created hydrolysis equation provides a strong approximation.

5.3.1.1 Modelling

In the three-factor test, which is used for non-linear functional relationships, each factor is tested at three levels. In general, it can be described by the following equation:

$$Y = b_0 + \sum b_L X_L + \sum b_Q X_Q^2 + \sum b_{12} X_1 \cdot X_2 \tag{5.1}$$

where,

- b_0: Zero-beat of the built model
- b_L: The optimized constant of the linear equation for the given parameter
- X_L: Physical quantity of the parameter under study (linear part)
- b_Q: The optimized constant of the quadratic equation for the given parameter
- X_Q^2: Physical quantity of the parameter under study (quadratic part)
- $\sum b_{12} X_1 \cdot X_2$: the interaction of each factor

Using equation 5.1, we can write the following equation for the 3^P factor test:

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 \tag{5.2}$$

To optimize enzymatic hydrolysis, the following equation is obtained, which is written according to the 3 influencing factors (enzyme concentration, hydrolysis temperature and hydrolysis time):

$$Y_{red.\ of\ Allergen} = 87.590 + 2.21\left(\frac{Enzyme\ conc.-0.08}{0.004}\right) - 0.282 * \left(\frac{Enzyme\ conc.-0.008}{0.004}\right)^2 + 0.06\left(\frac{Temp.-20}{10}\right) - 0.275\left(\frac{Temp.-20}{10}\right)^2 + 0.0271\left(\frac{Time-10}{5}\right) + 0.142\left(\frac{Time-10}{5}\right)^2 \tag{5.3}$$

Simplifying equation 5.1, we obtain:

$$Y_{red.\ of\ Allergen} = -17625(x_1 - 0.08)^2 + 552.5(x_1 - 0.08) - 0.00275(x_2 - 20)^2 + 0.006(x_2 - 20) + 0.00568(x_3 - 10)^2 + 0.00542(x_3 - 10) + 87.59 \quad (5.4)$$

Then, decomposing the simplified equation 5.3 in parentheses, we obtain:

$$Y_{red.\ of\ Allergen} = -176525 * x_1^2 + 834.5 * x_1 - 0.00275 * x_2^2 + 0.116 * x_2 + 0.00568 * x_3^2 - 0.10818 * x_3 + 41.5558 \quad (5.5)$$

where,

- x_1: Enzymatic concentration (g/L)
- x_2: Temperature (°C)
- x_3: Time (min)

It is now clear from the equation that both quadratic and linear relationships are present for hydrolysis from 3 factor. However, their effect is illustrated by the Pareto diagram in **Figure 20**.

5.3.2 Pasteurized and homogenized milk (2^P design)

In the measurements, a concentration of 0.012 g/L papain enzyme was used for hydrolysis.

5. Table The allergenicity level (AL) in the cow's milk (CM) with different fat content before the treatment

	CM 0.1%	CM 1.4%
AL in the milk before the treatment	35000	28500

6. Table Allergenicity level (AL) reduction after the treatments with different hydrolysis temperature, and -time

Temperature (°C)	Time (min)	AL in CM 0.1% (mg/kg)	AL in CM 1.4% (mg/kg)	AL reduction CM 0.1% (%)	AL reduction CM 1.4% (%)
40	10	40000	82 000	+14.29	+/187.72
40	15	51000	36 000	+45.71	+26.32
50	10	53000	41 000	+51.43	+43.86
50	15	40000	41 000	+14.29	+43.86
60	10	34000	69 000	-2.86	+142.11

In the 2^p study, homogenized and pasteurized CM was hydrolysed with two different fat contents, one 0.1% and one 1.4%. The enzyme papain was added to each sample at a concentration of 0.012 g/L, which was the optimization result obtained in the previous measurement. In this measurement, I tried to investigate the effect of hydrolysis time and temperature on allergen reduction in more detail, using a different type of heat-treated milk.

As can be seen from **Table 5**, the allergenicity decreased in only one case. However, for the two types of milk with different fat contents, it was observed that the percentage reduction in allergenicity for milk with a lower fat percentage increased the allergenicity level of the CM to a lesser extent than milk with a fat content of 1.4%.

During pasteurisation, milk is treated at a lower temperature than UHT, this can cause hidden (conformational) epitopes to remain unexplored and these are revealed during pre-inhibition and enzymatic hydrolysis of the protein structure, providing and IgE binding site.

Overall, it is also likely that there is a relationship between conformational epitopes and milk fat content.

5.3.3 Different treatments comparison

The enzyme concentration used was 0.012 g/L.

7. Table Times and temperatures of the different phases of enzymatic hydrolysis

	Time (min)	Temperature (°C)
Pre-heating	60	50
Enzymatic hydrolysis	15	50
Inhibition	30	70

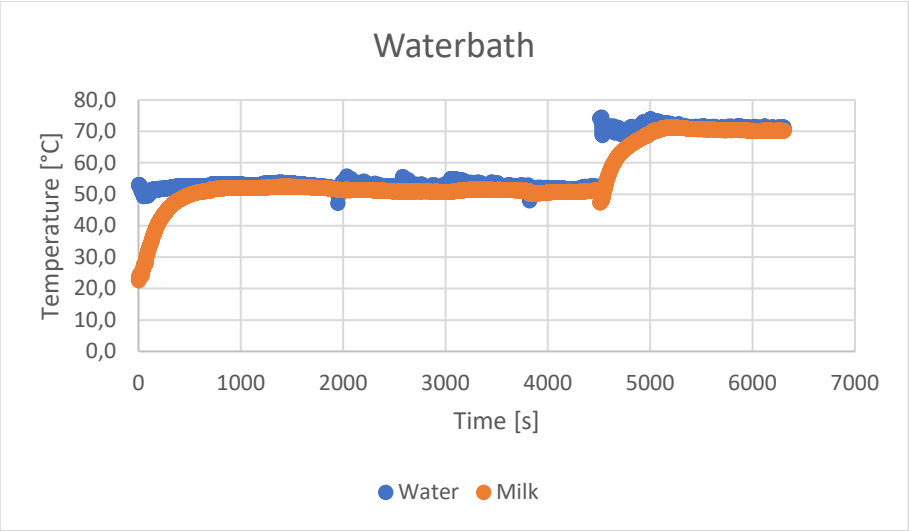
8. Table Comparison of CM allergenicity level after different treatment methods

Treatment	AL in CM (mg/kg)	AL reduction (%)
UHT Whole Milk	13 000	
Watherbath	18 000	+38.46
Ohmic heating	25 000	+92.31
Radio frequency	14 000	+7.69

Similarly in this scenario, a comparable rise in allergenicity level was observed. The most substantial increment was witnessed during the application of ohmic heating, wherein it is plausible that the interplay among milk proteins engendered by milk fat could be disrupted, thereby leading to the dissociation of various conformational epitopes binding. In the case of enzymatic hydrolysis performed in a water bath and pasteurized homogenized milk with a fat content of 1.4%, similar percent allergenicity reduction results were obtained. This makes it likely that, in case of higher fat content, more hidden epitopes remain in the cow's milk in case of certain treatments (pasteurization, UHT, ESL). Also, during the radiofrequency and ohmic heating, the secondary structure of the protein was broken down, so that hidden epitopes were released.

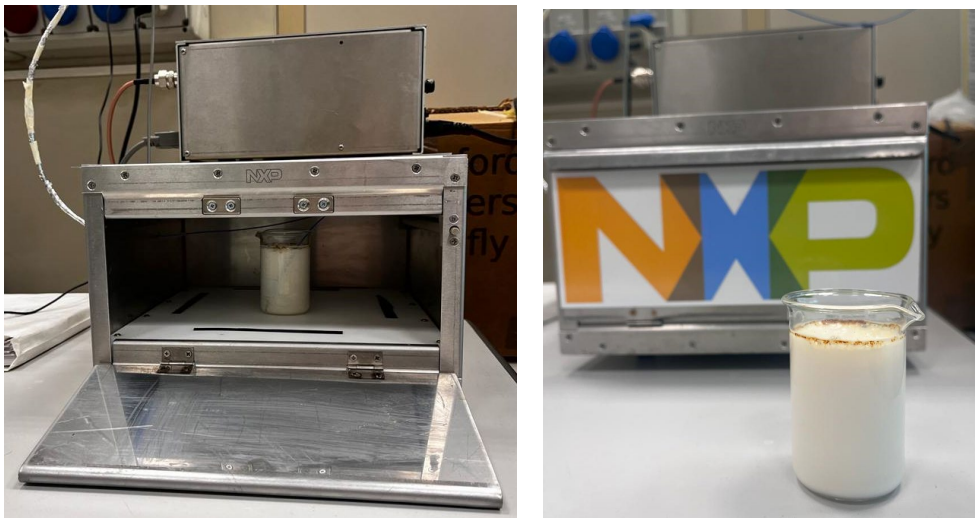
22. Figure Enzymatic hydrolysis in water bath and system for the temperature monitoring and control of individual samples and of water bath

During the enzymatic hydrolysis by heating the water bath, both the temperature of the individual enzyme+CM samples and the temperature of the heating water were monitored using optical cables.



23. Figure Temperature-time profiles of the water and the milk

In normal enzymatic hydrolysis, where the milk and enzyme complex were heated in water bath, a much more elongated heating cure is seen. Both the incubation temperature and the temperature of inhibition were reached by a curve similar to the exponential.

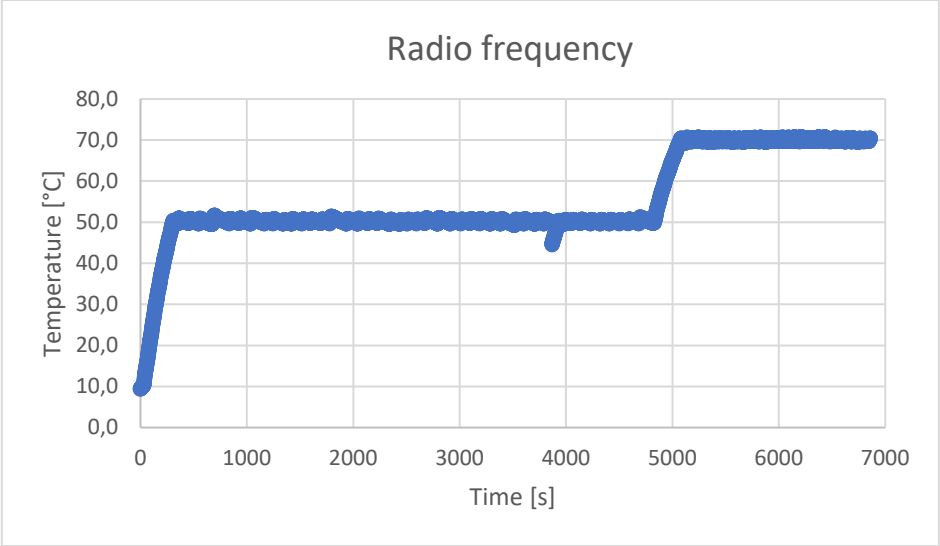


24. Figure Radio-frequency system used for the trial and the CM after the radiofrequency treatment

In order to heat the milk properly, a beaker had to be chosen in which 100 mL would fill it.

25. Figure Clearly visible physical changes in the milk

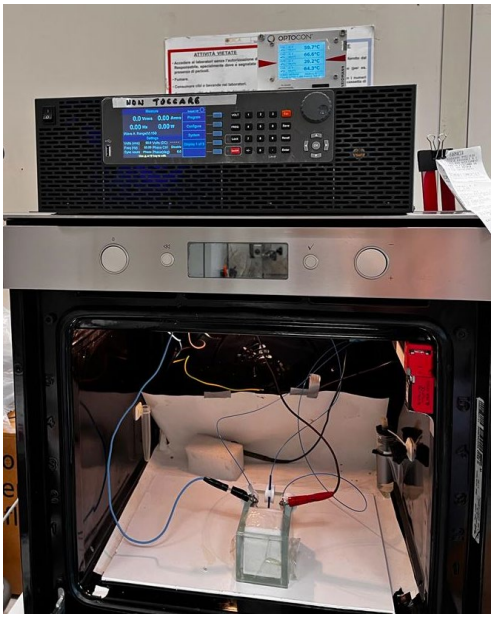
Figure 25 clearly shows that physical changes have occurred during the radiofrequency heat treatment. A brownish colour is observed on the surface of the milk, which is probably due to the Maillard reaction. Aggregates were formed throughout the milk, so that a separation of protein and whey was observed.



26. Figure Temperature-time evolution of the milk during the radiofrequency treatment

For CM heated by radiofrequency, the temperature reached the pre-incubation and inhibition temperatures linearly. The drop in temperature seen before 400 sec shows the enzyme inoculation required fir hydrolysis, as it was necessary to remove the beaker from the apparatus, causing a minimal loss of temperature. Compared to **Figure 29**, it can be observed that the temperature could be controlled much more evenly than during ohmic heating, much more

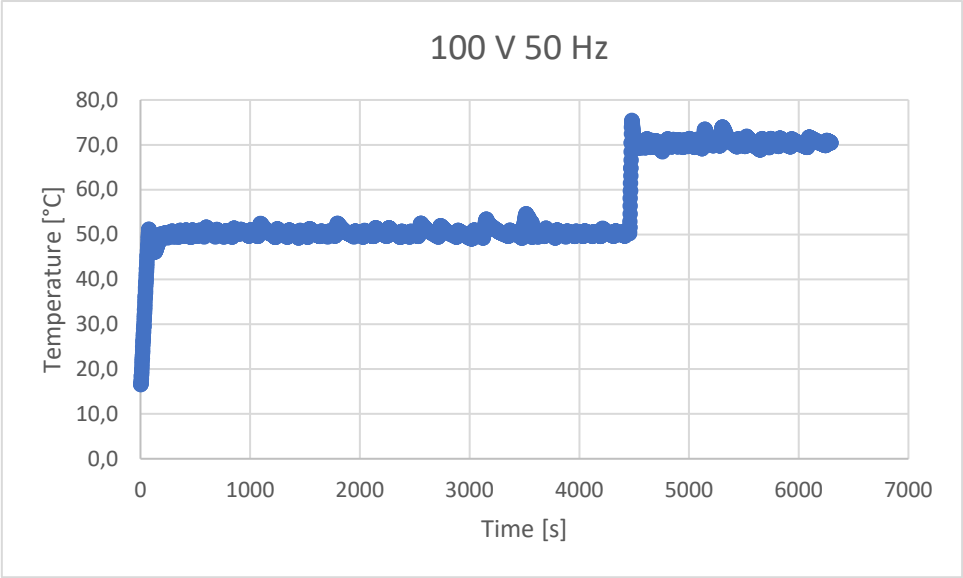
smaller temperature swings are visible. The retention curves at temperature of 50 and 70 °C are much more like the temperature change diagram of the enzymatic hydrolysis carried out in a water bath (**Figure 23**).



27. Figure The system of Ohmic heating treatment

28. Figure The electrodes with the fat and whey aggregates after the hydrolysis

In the present study, Maillard reaction and aggregate formation were also observed in the milk, as shown in **Figure 28**. But in this case, they were not found in the milk or on the milk surface, but on the electrodes in the cell. Oxidation lesions are also visible on the electrodes due to the pH of the CM.



29**. Figure** Temperature-time evolution of samples treated at 100 V 50 Hz in the ohmic heating system

The temperature-time evaluation profile plot shows a much steeper slope during heating, due to the higher power of Ohmic heating. The incubation temperature and the temperature of inhibition were achieved almost instantaneously with this type of heating. The fluctuations shown in the **Figure 29**. are due to temperature control After reaching the desired temperature, the system has still transferred energy to the cell to the cell by switching off.

6 Summary

Cow milk has become a vital component of our daily diet, appreciated for its nutritional value and appealing sensory qualities. Nevertheless, its allergenic nature poses a challenge for many individuals. Over the years, extensive research has been conducted to investigate the composition responsible for cow milk's allergenic properties and the processes associated with this allergenicity. With an improved understanding of allergenicity, numerous studies have been undertaken to mitigate the allergenic potential of cow's milk proteins. In pursuit of this goal, diverse treatment methods are employed to disrupts the bonds within milk proteins through chemical or physiological means.

The study outlines various treatment methods, including physical techniques like ultra-heat treatment, high hydrostatic pressure treatment, high-pressure homogenization, microwave treatment, ultrasound treatment, ohmic heating and radio frequency treatment, as well as chemical methods like acid treatment and enzymatic hydrolysis. These approaches show promise in reducing allergenicity in cow's milk, yet there are also debates regarding the suitability of some of these technologies.

Enzymatic hydrolysis is regarded by certain studies as the optimal approach for reducing allergens, in recent times, numerous experiments have been conducted with various enzymes to create allergen-free products. Remarkable outcomes have been achieved with trypsin; however, the production of this enzyme is more labour-intensive when compared to plant-based proteases.

In the current investigation, preliminary experiments indicated that papain was the most suitable enzyme for hydrolysing CMPs. Optimization experiments have been conducted by using enzyme concentrations between 0.008 g/L and 0.016 g/L, hydrolysis durations spanning 10 to 20 minutes, and hydrolysis temperatures ranging from 40°C to 60 °C. Reduction percentages of 86.7% and 90% were observed during the experiment. A 3^P factor optimization approach was employed to determine the ideal operating conditions, which turned out to be a concentration of 0.012 g/L, a temperature of 50 °C, and a hydrolysis duration of 15 minutes, considering the perception of bitter taste. For the further experiments, these working conditions were used.

In the „2P" factor experiment, which involved pasteurized and homogenized cow's milk with two different levels of milk fat content, there was an observed increase in allergenicity. This

increase was attributed to the fact that the cow's milk in this experiment received less heat treatment compared to the UHT skimmed milk used in the 3^P factor design. As a result, during the enzymatic hydrolysis process, conformational epitope bonds were released.

The third phase of the research incorporated different heating techniques alongside enzymatic hydrolysis to assess their impact on cow's milk. Ohmic and radio frequency heating methods were introduced in addition to the conventional water bath enzymatic hydrolysis. The results obtained based on the ELISA test did not show a decrease in the level of allergenicity, which may be due to the whole milk used. Additionally, physical treatments can disrupt the secondary structure of cow's milk protein, potentially leading to the exposure of hidden epitopes.

According to the findings at hand, a decrease in allergenicity was noticed in skimmed UHT milk. This underscores the influence of milk fat content on the results of enzymatic hydrolysis. Furthermore, alternative heating methods, like the ohmic and radiofrequency heating, hold promise for allergenicity reduction, although further experiments are needed to fine-tune the procedures and minimize physical alterations in the milk, such as aggregate formation, denaturation, and the Maillard reaction.

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List of tables and figures

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