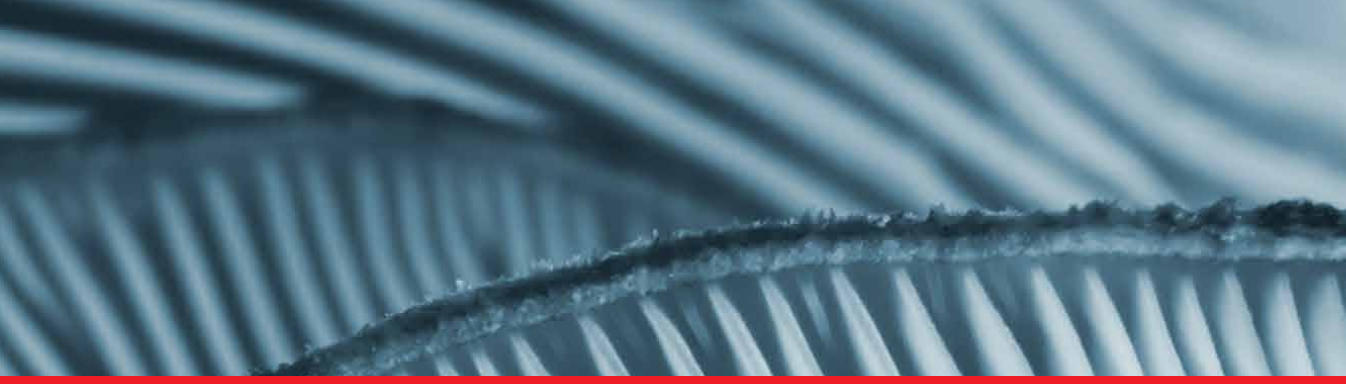

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Milk Proteins

Technological Innovations, Sustainability and Novel Applications

Edited by Jayani Chandrapala

Milk Proteins - Technological Innovations, Sustainability and Novel Applications

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Contributors
Alessandra Cutillo-Foraster, Ana Arnaiz, Bhimesh Ashritha, Channahalli Thammegowda Manoj Kumar, Eiji Kinoshita, Emiko Kinoshita-Kikuta, Jayani Chandrapala, Jorge Lucas Vallejo-García, José M. García, Juhi Saxena, Malladevanahalli Huchegowda Sathish Kumar, Marta Liliane de Vasconcelos, María Asunción Muñoz Santamaría, Miriam Trigo-López, Priyanka Singh Rao, Saúl Vallejos, Sonja Kukuljan, Todor Vasiljevic, Tugba Kilic, Tuyen Truong, Yuanyuan Zhao

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Food Science and Nutrition

Volume 7

Aims and Scope of the Series

The significance of food is undeniable, especially in light of the impending challenge facing humanity: ensuring there will be enough food to meet the basic needs of a population expected to reach approximately 10 billion by 2050. These food-related challenges align with some of the United Nations' sustainable development goals, with a target to achieve them by 2030. One thing is certain: food should be not only nourishing and safe but also tailored to the diverse needs of individuals throughout their lifetimes, all while meeting consumers' sensory expectations. Understanding the diverse chemical composition of food, often referred to as biodiversity, and how these components can contribute to human health by considering factors like bioaccessibility, bioavailability, and bioactivity at the organ level, is crucial for grasping and promoting a healthy diet. Thanks to the continuous evolution of analytical methods and interdisciplinary research, significant strides have been made in the field of food science and nutrition.

Meet the Series Editor

Maria Rosário Bronze has been working in Analytical Chemistry since 1986. Her Ph.D. in 1999 contributed to the study of food products using capillary electrophoresis. The main goal of her research since 1999 has been focused on Analytical Chemistry applied mainly to the analysis of foods and by-products of food industry. She conducted research in collaboration with national and international research groups, at iBET and ITQB Technology Division. From 2017 until 2021 she was head of Food & Health Division at iBET and head of the Food Functionality and Bioactives Laboratory. MR Bronze has been an Associate Professor at the Pharmacy Faculty of Lisbon University and head of the Structural Analysis Laboratory since 2012. As a researcher, MR Bronze is a Senior Scientific Advisor at Food & Health Division at iBET and Head of Food Functionality and Bioactives Laboratory at the same Institute, Collaborator at iMED and Researcher at ITQB NOVA. Her current research is focused on quality and beneficial health effects of food components. Gas and liquid chromatography associated with mass spectrometry are used by MR Bronze in the characterization of samples. Sensory evaluation is also an important area of her research. The main food products studied by her are olive tree products (olive, olive oil, leaves), cereals such as maize, legumes (faba bean, pea, chickpea, lentils) fruits (apple, grapes, opuntia ficus), fruit juices and wine, among others. More recently her interests have also involved biodiversity, bioaccessibility, and bioavailability studies on food products and their components, mainly phytochemicals as phenolic compounds, using different analytical tools such as mass spectrometry. As a senior scientific advisor at Food & Health Division at iBET she is involved in different areas: (i) isolation, characterization and formulation of bioactive and functional compounds or extracts from natural sources and wastes from food and other related industries; (ii) pre-clinical assays to provide support to understand health claims related with the beneficial effects of food nutrients/bioactive components; (iii) establishment of analytical methodologies including mass spectrometry state-of-the-art to fully characterize different matrices, from food products, natural extracts or biological fluids (Food Functionality and Bioactives Laboratory).

Meet the Volume Editor

Jayani Chandrapala is an associate professor at RMIT University, Australia, with international recognition in food science and technology. With nearly 170 publications, she serves as an associate editor for three leading journals in the field and part of the editorial board for four others. Her expertise includes the physical chemistry of food systems, protein conformational changes, component interactions, membrane processing for waste valorization, and emerging non-thermal technologies. Assoc. Prof. Chandrapala is ranked #4 in the AD Scientific Index from RMIT, #29 from Australia, #51 in Oceania, and #740 in the world in food science and engineering.

Contents

Preface

Milk proteins and their influence on other components of milk are at the forefront of research, offering immense potential across diverse industries. This book explores recent milk protein research, highlighting key advancements in extraction, preservation, and functionality modification techniques. Furthermore, it presents insights into processing and modification techniques, including heat treatment, enzyme treatments, and hydrolysis. Driven by environmental and sustainability concerns, the research has expanded to explore sustainable, convenient practices, taking significant steps towards greener dairy processing. The applications of milk proteins now extend far beyond traditional dairy products, finding new roles in pharmaceuticals, cosmetics, and even baked goods.

I would like to extend my heartfelt thanks to the publishing processing manager, whose guidance and support have been invaluable throughout the development of this book.

Jayani Chandrapala
School of Science,
RMIT University,
Melbourne, Australia

Chapter 1

Extraction of Bioactive Peptides from Whey Proteins by Conventional and Novel Technologies

Tugba Kilic

Abstract

Bioactive peptides show physiological properties in systems such as digestive, cardiovascular, and vascular. Bioactive compounds are found in animal and plant proteins. However, peptides obtained from milk proteins have better biological activity in terms of amino acid composition and sequence. It is important to industrially evaluate bioactive peptides after proper extraction, purification, and identification. There are conventional (enzymatic hydrolyzation and fermentation) and novel methods (ohmic heating, ultrasound and microwave) for the extraction of bioactive peptides. Novel extraction methods increase the degree of hydrolysis of peptides, making them more efficient, and peptides with high activity are obtained. The extraction method of bioactive peptides to be extracted from whey is important, and the method to be chosen must be evaluated in all its aspects. This chapter includes literature data on the importance of whey proteins, bioactive peptides, and extraction methods of bioactive peptides from whey.

Keywords: whey proteins, bioactive peptides, hydrolysis, extraction, novel technologies

1. Introduction

There is a growing scientific and industrial interest in natural and generally side-effect-free bioactive compounds. Reducing the risk of chronic diseases is possible through the bioactivity of peptides with physiological properties [1]. The functions of bioactive peptides (2–20 amino acids and <10 kDa) vary depending on amino acid composition, charge, sequence, and molecular weight [1–3]. Peptides can be obtained from animal and plant proteins. In milk, which is an important source of peptides, bioactivity varies depending on the joint effect of one and/or more peptides [4, 5]. The majority of milk-derived bioactive peptides are composed of casein (α, β, γ, κ-casein) and precursors of serum proteins [2, 4]. In addition to peptides derived from casein such as immunopeptides, cytokinins, casomorphins, and casoxins, there

are peptides derived from serum proteins such as α-lactorphin, β-lactorphin, lactoferricin, lactoferroxins, phosphopeptides [2].

Whey (approximately 80–90% of milk), which is the remaining part of casein after coagulation in cheese production, is a yellowish-green liquid with a yellowish/ waste product due to riboflavin [6, 7]. Whey becomes a risk for many living things due to the effect of microorganisms operating using organic matter and oxygen in the environment [7]. Due to its high oxygen demand, it is important to process whey and use the proteins it contains in various industries [8]. Many products (powder, protein, enzyme, and various acids) are obtained from whey by using physicochemical (spray drying, thermal/isoelectric precipitation, membrane filtration, coagulation) and biotechnological processes [9]. While the water content of whey is reduced, the protein content is increased, resulting in products with a longer shelf life and more nutritious products [10, 11]. The solubility (hydrogen bonds and surface hydrophobicity), gelling properties (sulfhydryl groups and disulfide bonds), and emulsifying properties (surface activity) of proteins vary depending on the different properties in their structures [9, 12]. Whey proteins are used in the production of foods thanks to their textural and sensory properties such as gelling, foaming, and fat binding, as well as improving nutritional quality with protein content [7]. Whey products are used in many products such as beverages, bakery, milk, meat, and sports foods [11]. Whey biofilms of fruits and vegetables reduce respiratory rate and microbial activities and extend their shelf life [13]. β-Lactoglobulin (β-LG), which is not found in human milk [14] but makes up a large proportion of whey proteins, is an allergen for infants [15]. For this reason, whey enriched with α-lactalbumin (α-LA) and lactoferrin (LF) is used in infant foods [13].

It is clear that whey is not made up of simple proteins, but is a complex structure made up of many bioactive peptides with their unique effects. The applied technologies are of great importance in the activity of these biopeptides [13]. The type, number, quantity, and properties of peptides obtained from whey proteins vary depending on the method used and its conditions. Conventional methods (fermentation, enzymatic hydrolysis, *in vitro* digestion, and chemical/thermal application) are used in the dairy industry to isolate peptides. Peptides in whey show individual bioactivity, but conventional methods used to separate each peptide are inadequate. Because peptides and their biological properties vary depending on the enzyme and microorganism or conditions used in the extraction method. Traditional methods also have disadvantages such as being costly and taking a long time to hydrolyze proteins. With the development of these methods or the use of a novel method, more peptides are obtained and the functional properties of these peptides are increased. With novel methods, costs are reduced, and faster and bioactive peptide production takes place [2, 3]. However, the inadequacy of some novel methods in obtaining peptides and their application before or at the same time as conventional methods contribute to peptide extraction. This book chapter will focus on extracting peptides from whey using old and novel methods.

2. Whey proteins/peptides

Whey, also known as serum proteins or soluble milk proteins, consists of water, lactose, whey proteins, minerals, milk fat, and vitamins [8, 16]. However, whey proteins are not correctly called serum proteins because they contain glycomacropeptide (GMP), which is both partially free in milk and formed by hydrolysis of casein [6].

Whey is effective in growth and development because it contains proteins and amino acids with high biological value [17]. The main proteins are β-LG (50–55%), α-LA (20–25%), GMP (10–15%), immunoglobulins (IGs, 10–15%), bovine serum albumin (5–10%), LF (1–2%), lactoperoxidase (LPO, 0.50%), and lysozyme (0.002%) [18]. Whey proteins contain many essential (such as cysteine and methionine) and nonessential amino acids [13, 19]. The content of whey varies depending on the quality of the milk, processing, and purification method [8]. The pH of the milk decreases with microbial growth or direct acid addition and the casein coagulates, thus producing sour/acid whey (max pH 5.1). The product formed as a result of coagulation of milk with the addition of enzymes is yeast/sweet (min pH 5.6) whey [10, 20, 21]. Sour/acid whey is nutritionally superior to yeast/sweet whey [10, 20].

Peptides can exert both synergistic and antagonistic effects. For example, Lf may have antimicrobial properties from the synergistic activity of defense proteins such as LPO and lysozyme [16]. Bioactive peptides can exhibit more than one different biological activity [22]. Whey proteins/peptides have antioxidant, anticarcinogenic, antihypertensive [7, 13], immunomodulating, mineral-binding, antiobesity [13], antimicrobial activity [13], antidiabetic [13, 16], opioid agonist, and antagonist effects in *in vitro* and *in vivo* conditions [23]. Whey proteins also have positive effects such as prevention of muscle degeneration, initiation of wound care and repair, benefits for infant nutrition, and healthy aging [13].

Peptides exhibit antioxidant properties due to their radical scavenging effects on the activity of enzymes in the cell [23] and the inhibition of enzymes such as xanthine oxidase [24] or by binding to prooxidant metal ions [1]. Some amino acids (cysteine, histidine) can bind free radicals by transferring hydrogen atoms or electrons [1]. Whey proteins also contain amino acids such as methionine and cysteine, which show antioxidant activity as a precursor to glutathione [25]. In cysteine, especially the sulfhydryl group is responsible for antioxidant activity [16]. LF, which binds iron, reduces the conversion of hydrogen peroxide to the hydroxyl group [13, 25]. Many peptides identified from whey have antioxidant activity [16, 22].

Opioid peptides have agonistic activity by binding to opioid receptors (μ-, δ-, and κ-type) and acting on antagonistic or N-terminal sequences. Opioid agonistic peptides such as α-lactorphine, β-lactorphine, and serorphine [16] have a drug-reducing and/or -inhibiting effect, while opioid antagonistic peptides such as lactotensin and lactofotoxin A and B have a reducing and/or inhibiting effect [5].

Bioactive peptides have an immune-boosting effect thanks to their effects such as antibody synthesis, cytokine regulation, and lymphocyte proliferation [1, 16]. However, the amino acid content, charge, length, sequence, and hydrophobicity of immune-acting peptides are important [1]. For example, LF with its positively charged parts [26], GMP with its effect on lymphocytes [13], and LF with lipoprotein-binding properties compete with viruses [27], β-LG containing sulfurous amino acids [6] and IGs [28] have immune system-supporting properties. Whey proteins contribute to immunity, especially in newborn babies, by reducing allergic reactions [16]. Glutathione deficiency in individuals with HIV was eliminated by the consumption of β-LG, α-LA, and LF, strengthening the immune system of these individuals [7].

Peptides in whey protein structures are effective on angiotensin I-converting enzyme (ACE, peptidyl dipeptide hydrolase, EC 3.4.15.1), which is a key antihypertensive [16, 29]. In addition, the ACE inhibition (ACEi) activity of peptides increases the mineral density in the bones and has a positive effect on bone health [30]. Whey peptides such as α-lactofores, α- and ß-lactorphins [31], albutensin [5], β-lactosin [32], and ß-lactokinins have ACEi effects [31]. Amino acid residues at the

C (aromatic) and N terminal (hydrophobic) of peptides are effective in ACEi [1]. α-Lactorphine, whose N-terminal dipeptide is Tyr-Leu, has the highest ACEi activity among whey peptides [29].

Whey proteins, which increase the amount of glutathione and/or bind iron [7], have an anticarcinogenic effect by contributing to immunity [25]. In addition to preventing colorectal cancer by binding iron [33], LF has effects such as reducing anemia in chemotherapy patients [34]. α-LA and β-LG reduce the risk of developing colon and breast cancers [7]. Whey peptides are an important antiproliferative agent [16].

IGs, LF, and LPO have been shown to have antimicrobial properties from PAS proteins [35]. The positive charge of LF [36], its hydrophobic structure, and its secondary structure are thought to support its antimicrobial properties. LF contributes positively to the activity of antibiotics by increasing the permeability of the cell wall by binding microorganisms to its outer membrane [14]. LF, which has a stronger antimicrobial effect than LF, increases membrane permeability with the basic amino acids it contains [36]. LPO becomes a natural antimicrobial agent through the oxidation of thiocyanate with hydrogen peroxide [31].

Whey proteins have a positive effect on the control of insulin secretion and blood sugar. This effect is because peptides can induce insulin secretion [22].

Metal-chelating peptides improve divalent mineral bioavailability and do not bind cations such as calcium, iron, and zinc [16]. β-LG binds minerals and transports them along the intestinal walls [6]. Thanks to its iron-binding properties, LF plays an inhibiting role in microbial activities and oxidative reactions and supports bone development [13, 37]. Four peptides from β-LG and 2 peptides from α-LA have mineral-binding effects [16]. GMP [5] and lactotransferrin have antithrombotic effects by binding to receptors in platelets [36].

β-LG can bind vitamin A (retinol) and vitamin D due to its capacity to bind with a large number of hydrophobic and amphiphilic ligands due to the presence of calyx in its structure and hence can act as a carrier for them. It is also involved in the stimulation of lipase activity [6] and phosphorus production due to its ability to bind β-LG fatty acids [37]. α-LA, a good calcium carrier, is involved in synthesizing lactose [38]. α-LA reacts with β-1,4-galactosyl transferase-1 and increases its affinity for glucose by a factor of 1000, thus enabling more lactose to be produced [6]. Whey proteins provide long-term satiety with a low glycemic index and regulate glucose homeostasis levels. The leucine they contain provides fat loss and helps weight loss [7].

3. Extraction of bioactive peptides

Whey proteins, which are primary, secondary, tertiary, and quaternary under certain pH, ion strength, and temperature conditions, denatured with chemical, enzymatic, and physical processes, change their structure and physicochemical properties. Reactive amino acids and whey proteins, which emerge due to the changing structure, cluster depending on the pH. Since whey proteins have different molecular properties, structures, and denaturations, these factors change when they are found together. For example, when α-LA and β-LG are together, denaturation temperature and aggregate sizes are affected [39]. The quaternary structure of β-LG, consisting of 162 amino acids, varies with low concentration (<10 mg/mL) and high pH at temperature (<10°C). It is in the form of β-LG monomer at very low pHs (pH<3.4) and high pH (pH>8). β-LG exists as non-covalently linked dimers between pH 5.2–8.0 and as octomers at pH 3.5–5.2 [8]. With increasing temperature (65°C≤), β-LG denatures and

aggregation of oligomers causes irreversible aggregation, releasing free sulfhydryl. However, in skimmed milk with a high water content, β-LG denatures to 75–100°C at higher temperatures [39]. α-LA (about 14 kDa), on the other hand, is less sensitive to pH changes [8].

Bioactive peptides are in inactive form but become active by the activity of digestion and commercial enzymes or by fermentation [16, 22, 36, 40]. The number, sequence, and type of amino acids in peptides and the carboxy or amino terminus are important in peptide activity [4]. In addition to microbial and enzyme activities [4], many different peptides are activated chemically by acid/base hydrolysis [20]. Additionally, when these are used in combination, shorter peptides are obtained [16]. Novel technologies include ohmic heating (OH), microwave-assisted extraction (MAE), pulsed electric fields (PEF), subcritical water hydrolysis extraction (SWE), ultrasound-assisted extraction (UAE), and high hydrostatic pressure (HHP) (**Table 1**). Conventional methods alone can be used to produce peptides. However, fermentation or enzymatic hydrolysis after some novel technologies (UAE, MAE, and HHP) can be used as pretreatment for peptide recovery [2, 3, 19].

3.1 Conventional methods

3.1.1 Enzymatic hydrolysis (EH)

Biological catalyst enzymes are protein structures that show activity inside or outside the cell. The most commonly used method in protein hydrolysis is enzymatic [16]. EH contributes to the improvement of properties of proteins such as nutrition, functionality, emulsification, solubility, foaming, and gelling [53]. The degree of EH is determined as a percentage depending on the peptide bonds [13]. There is a direct relationship between the degree of hydrolysis and the activities of bioactive peptides [3]. EH takes place under controlled conditions (hydrolysis time, pH, temperature, enzyme/substrate ratio, and enzyme activity) and is an effective method of obtaining peptides [2]. The enzymes used for this purpose can be of animal, vegetable, microbial [2], and digestive (chymotrypsin, trypsin, alkalase, pancreatin, thermocillin, and pepsin) origin [16]. When these enzymes are used in combination, more peptides are formed and the activity varies according to the order of the enzyme used [16]. Plant-derived proteases are widely used in the production of peptides because they operate in wide temperature and pH ranges [54]. They can be of animal, plant, or microorganism origin with some enzymes. For example, transglutaminase has high activity at certain temperatures (≈50°C) and over a wide pH range (pH 5–8) [13].

To understand the hydrolysis of proteins during digestion, it has been shown that proteins are broken down by pancreatic proteinases (mostly trypsin), and many peptides are released *in vitro*. In addition, combinations of proline-specific endopeptidase, thermolysin, elastase, carboxypeptidase, pepsin, pancreatin, and chymotrypsin endoprotease can also produce bioactive peptides [2, 3]. Trypsin is a digestive enzyme used especially in obtaining peptides showing ACEi activity [16].

Pepsin is used to obtain peptides with bioactive properties different from α-LA (α-lactofophorin, α-lactorphine), β-LG (β-lactofophorin), serum albumin (albuntensin, serrorfin), and lactotransferrin (thrombin inhibitor peptide, casonplatelin). Trypsin produces peptides with different bioactive properties from α-LA, β-LG, β-lactoxine, and GMP. Enzymatic hydrolysis of bovine whey with α-LA with trypsin and alkalase formed the peptides LDQWLCEKL, ELKDLKGY, and ILDKVGINY, respectively [19].

		Advantages	**Disadvantages**
Conventional	EH	High specificity [41] Elimination of organic solvents as per [41] Non-toxicity of the resulting product [3]	A limited number of enzymes are available [41] Costly [2, 41] Low yield [41] Bitterness [2]
	Fermentation	Elimination of multifunctional peptides [42] Release of exopeptidases that hydrolyze N-terminal amino acids [43] Natural and controlled conditions [2] Relatively economical [41]	Depending on the microorganism strain, the degree of hydrolysis and functional properties vary [43] Peptide production is low and specificity is low [2, 41]
	Chemical/ thermal	Simple and cheap [41]	Difficult to control the process [41] Low peptide purity [18] Low functionality [41] Solvent need [41] Formation of undesirable compounds [41]
Novel	UAE	High energy efficiency [41] Environmentally friendly [1] Non-thermal [1, 2] High extraction selectivity [2] Acting on covalent bonds [13]	Temperature rise during application [41] Insufficient for peptide extraction alone [41]
	MAE	Simple to implement [41] Low cost [41] Fast and uniform heating [1] Easy to maintain [1] Repeatability [2]	
	HHP	Without damaging bioactive compounds [41] When applied in combination with heat treatment, hidden peptides are also revealed [19]	No effect covalent bonds [41] Denaturation may occur due to pressure [41] High cost of infrastructure [41] Failure to implement at large scale [41]
	PEF	Environmentally friendly [12] Non-thermal [25] Low energy needs [41] Short implementation time [41] Conformational effects on the secondary structures of proteins [41]	High investment cost [41] Thermal aggregates may form due to temperature increase [13, 44]
	OH	Improvement in bioactive properties [45] Uniform heating [41, 45] Less time [41] Energy conversion is efficient [41]	High investment cost [41] Application only in liquids/solids with solid particles [41] The rapid increase in temperature due to conductivity [41]
	SWE	Breaks peptide bonds when applied alone [41] Elimination of low molecular weight peptides [46] Green technology [41] Non-toxic water and no solvent residue [41] Improving efficiency with additives [47] The resulting peptides can be used without purification [41]	No specificity in peptide modifications [41] Industrial application limited [47] Infrastructure costly [41] Pre-treatment such as alkylation may be needed [41] High energy requirement [46] Toxic substances can be formed [46]

		Advantages	**Disadvantages**
The novel/ conventional technologies	UAE-EH	Change of peptide profile [48] Shortening of hydrolysis time [48, 49] Low molecular weight peptide extraction [48, 49] Increase in bioactivity [48] Reducing the amount of enzymes required [48] Increasing substrate sensitivity [39, 41]	
	HHP-EH	Increased number of bioactive peptides [50] Enhancement of physiological properties [51] Enzyme to improve substrate accessibility [41]	After HPP pretreatment, protein/peptide interaction may occur again [3]
	MAE-EH	Obtaining low molecular weight peptides [41] Enables rapid and efficient hydrolysis [2, 52]	
	OH-EH	Enhancing bioactive properties [48]	

Table 1.
Extraction technology of whey bioactive peptides.

Whey proteins are difficult to hydrolyze due to their spherical structure. Therefore, pretreatment (physical/chemical denaturation) is required, and the number of peptide bonds increases, making enzymatic hydrolysis easier. By pretreatment, the main proteins in the whey are broken down and the hydrophobic parts are collected on the outer surface so that the peptide bonds become suitable for enzymes. However, the functionality of the peptides varies depending on the pretreatment used. In addition, if sulfitolysis, which is a reversible reaction with the SS bonds of sulfite, is used as a pretreatment method for whey proteins, it increases the degree of enzymatic hydrolysis since heat and pressure are not applied [55].

3.1.2 Fermentation

In the production of dairy products, many different bioactive peptides are formed by biochemical changes during fermentation under natural or controlled conditions [16]. Lactic acid bacteria (such as *Lactococcus lactis*, and *Lactobacillus helveticus*) are usually used for whey, as well as nutrients, aroma, and physical healing [2] also occur in peptides [16]. The efficiency of fermentation, the degree of proteolysis of proteins, depends on the type of bacteria and fermentation conditions [2].

As a result of the fermentation of whey with 34 different lactic acid bacteria (48 h, 37°C), many peptides were obtained from B-LG and *Pediococcus acidilactici* SDL1414 showed the strongest ACEi effect, especially in peptides with low molecular weight [43].

3.1.3 Chemical/thermal methods

The chemical hydrolysis of whey proteins is related to sulfides in the structures of proteins. The function and properties of proteins can be enhanced by lowering the pH

with succinic acid or by influencing disulfide bonds by sulfitolysis [25]. When whey was brought to pH 3.0–4.6 with citric acid, high and soluble β-LG was obtained [18]. Chemicals such as phosphoric and hydrochloric acid can be used for hydrolysis. If acid hydrolysis and microwave are used simultaneously, the hydrolysis time is shortened [46].

When heat is applied to whey proteins, free thiol groups are released and denaturation occurs. Free thiol groups form aggregates as thiol-disulfide. Calcium facilitates the thermal precipitation of whey proteins. Generally, dehydrated whey is denatured at neutral pH at 90°C and then precipitated at pH 4.4–5.0 to obtain efficient but low solubility protein, which is important for functionality. For this purpose, it is recommended to first acidify with the addition of organic or inorganic acid (pH 2.5–3.5) and then apply heat treatment (90°C). The addition of iron contributes to solubility [56]. Seventeen peptides were obtained as a result of the treatment of β-LG with heat and lysine using different concentrations of calcium [57].

3.2 Novel extraction methods

Novel extraction techniques are based on physical processes used in the production of bioactive peptides to improve the degree of hydrolysis [2, 58].

3.2.1 Ultrasound-assisted extraction

The new UAE provides many benefits by using high frequency (≈20 kHz) waves that humans cannot perceive [1]. In the UAE method, ultrasound waves consist of mechanical waves propagating in an elastic medium. The cavities formed in a high-intensity sound wave are called cavitation bubbles [59]. At the cavitation point, temperatures increase locally and these cause chemical changes by producing radicals [44]. If the bubble is large, it will float and burst on a surface; if it is small, it explodes into the liquid [59]. This explosion can create the energy necessary for physical, chemical, and biochemical effects [13, 60]. Chemical effects can be seen at a frequency of 300–500 kHz, and physical effects can be seen at a frequency of 20 kHz [2]. Protein structures undergo conformational changes due to these effects; proteins are fragmented, and the free sulfhydryl content increases [2]. The viscosity and surface hydrophobicity of whey protein change in proportion to the applied energy density, and the particle size decreases [44]. Ultrasound can be applied using two equipment: an ultrasonic bath and a probe [61]. Ultrasonic baths generally operate at a frequency of around 40 kHz, but they get hot while operating [62]. Additionally, if the local temperature increases the temperature of the entire solution, proteins may denature. Since the rate of increase of this temperature depends on time, frequency, volume, and power, the UAE application needs to be optimized. Additionally, the temperature can be controlled during the process by adding ice, a cooling jacket, or by constantly changing the medium [44]. Depending on the frequency range, it can be applied as high (16–100 kHz, 10–1000 Wcm^{-2}) and low intensity (100 kHz–1 MHz, 1 Wcm^{-2}>). Low-intensity ultrasound is used in the food industry to ensure high quality nondestructively, while high-intensity ultrasound is used for material modification such as emulsification, microbial inactivity, extraction of flavors, protein modification, gas, and defoaming [13].

The number of disulfide bonds increases with hydrophobic interaction with the heat treatments applied to obtain peptides from whey proteins and the proteins are denatured. It can be used for many purposes such as solubility, gelling, and foaming

properties by ultrasound application in whey [13, 44]. Furthermore, cavitation bubbles induce induction and conformational changes in protein substrates. Thus, UAE reduces α-helical structure and increases β-sheet structures [48]. By reducing the size of fat particles coated with whey proteins, UAE increases the area of action of enzymes and allows more peptides to be obtained [2]. It has been shown that UAE administration of whey proteins before hydrolysis with alkalase shows ACEi and immunomodulatory activities depending on peptides. Whey proteins and peptides obtained after enzymatic hydrolysis with bromelain first (probe, 500 W, 20 kHz, 10 min) have low molecular weight (5 kDa>) and high ACEi activity [48]. Similarly, the conditions of an application before enzymatic hydrolysis (pepsin and papain) with an ultrasonic sonicator (300–400 W, 20 kHz, 2–4 min) and the degrees of hydrolysis vary according to the enzyme used [49]. β-LG, high-intensity ultrasound (frequency of 20 kHz, 60 Wcm^{-2}) has been reported to increase the sensitivity of pepsin and trypsin to proteolysis [39].

3.2.2 Microwave-assisted extraction

MAE uses microwaves with electromagnetic waves of different wavelengths (0.001–1 m) and frequencies (300 MHz–300 GHz) [1]. MAE extends the shelf life of foods and improves various functional properties [2]. The microwaves have positive effects on the taste and quality of products [1]. The power, frequency, time, and temperature to be applied in MAE are important [12]. Microwave energy causes molecular interactions involving ionic conduction and dipolar rotation mechanisms [2]. Changes in the structure of proteins occur by generating heat with the energy released by interaction at the molecular level [12].

It can be microwave, thermal, or noninductive, using nonionizing radiation. Thermal effects with microwaves arise from local heat generated by the friction of water molecules, while nonthermal effects arise from the rate of unfolding and rearrangement of proteins [2]. Because microwaves break down disulfide bonds in proteins, proteins open up [1]. This opening in milk proteins facilitates the application of enzymatic hydrolysis as in UAE. MAE (532 W, 40–50°C, 5 min) application of bovine serum albumin concentrate before proteolysis (such as pronase, chymotrypsin, papain, alcalase) increases enzymatic hydrolysis [2].

3.2.3 High hydrostatic pressure

HHP, also called "pascalization" or "cold pasteurization" [3], is a green new technology that covers the application of hydrostatic pressure (100–1000 MPa, max 30 min) with and without heat treatment. Depending on the HHP processing time and pressure, the temperature of the product increases optimally [2]. Proteins are denatured as the volume decreases with the applied pressure causing biochemical changes in the systems [44]. Unlike thermal treatments, HHP acts more on weak chemical bonds (hydrophobic, hydrogen, and ionic), activating only the secondary structures of proteins. The application of low pressure (<400 MPa) increases the hydrogen bonds, while high pressure has the opposite effect on these bonds [3]. HHP can be applied intermittently, semi-continuously, and continuously [2]. Denaturation and/or aggregation occurs depending on the HHP conditions (pressure, temperature, and time) and the proteins (pH and composition) [13, 44]. At high pressures, β-LG aggregates through disulfide bond exchange, particle size increase, formation of aggregates, and conformational change. At low pressures (<120 MPa), partial

opening of β-LG is observed with changes such as particle shrinkage and an increase in sulfhydryl groups [44].

When applied as a pretreatment, HPP can increase the efficiency of enzymatic hydrolysis (active site formation, chemical bonding, and interaction) by destabilizing whey proteins. For example, EH made using chymotrypsin, pepsin, and trypsin produced more hydrophobic peptides than β-LG pretreated with HHP [3]. Factors such as HHP conditions (pressure, temperature, and time), pH, ionic strength, substrate, and enzyme affect enzyme hydrolysis [2, 3]. In addition, hydrolysis should be performed without wasting time after HHP administration so that the susceptibility to proteolysis does not decrease. After HPP application, reaggregation of proteins may occur through protein and peptide interactions. When hydrolysis is performed simultaneously with HHP, protein agglomeration is prevented [3].

HHP contributes to the foaming properties as a result of increased opening and adsorption rates of proteins through intermolecular interactions at neutral pH. However, the application of pressure above 300 MPa reduces the stability of the foam by opening the protein [13]. In HHP application, it causes β-LG and α-LA denaturation of pressure higher than 100 MPa and makes it difficult to separate into fractions. β-LG has reversible effects on certain pressure (300 MPa>) applications [25]. Following the application of batch HPP (0.1, 400 and 600 MPa, 10 min, at room temperature) to bovine whey, QEAKDAFLGSF and WENGECAQKK peptides were obtained from β-lg by tryptic hydrolysis, and while the best yield was obtained with 400 MPa, 600 MPa pressure reduced their amount. Before β-LG chymotrypsin administration, HHP (400 MPa) showed longer and more hydrophobic peptide [2].

3.2.4 Pulsed electric field

PEF is used specifically for the inactivation of microorganisms [25] and enzymes [1]. PEF, which is a nonthermal process, generates a high-voltage field (≈50 kV/cm) with short pulsed electricity (20 μs>) of different alternating currents (10–100 kV) [25]. Process factors such as electric field strength and flow rate are important in PEF application [13]. Factors such as proteins (such as solubility, composition, molecular weight, hydrophobic groups, and conductivity), pH, temperature, viscosity, and ionic strength are also important [12]. PEF improves the properties of proteins by optimizing these factors [13]. Generally, short-pulse electricity application is more advantageous [44] and free radicals are formed by the polarization of proteins and the energy released. With the interaction of these free radicals, the dielectric constant of the proteins increases and the proteins open [12]. If the duration of the electrical pulse is prolonged, the opposite effect occurs and protein aggregates can form. In addition, the increase in protein aggregation and surface hydrophobicity at high temperatures (≈70°C) in the PEF was mostly attributed to the thermal effect. The effect of β-LG application time is greater than the electric field intensity [13].

Although it is known that the effect of PEF on whey proteins varies depending on the electric field intensity, there are many contradictions in the literature regarding this. Some researchers have concluded that PEF therapy does not effect on proteins [12]. However, the functional and structural properties of peptides can be improved by optimizing electric field density and processing time in PEF application in whey proteins [63]. In addition, thermal pretreatment with PEF may have a positive effect on the opening of proteins [12].

3.2.5 Ohmic heating

OH, which is a thermal process, can be used for pasteurization. OH uses alternating electrical currents [1]. OH, which takes its name from Ohm's Law (current, voltage, and resistance), is known by names such as joules, electrical resistance, direct resistance, and electro conductive heating. In this method, heat is generated by passing alternating current through the electrodes and resisting the conductive product against this current. The effectiveness of the method is related to factors such as voltage (transformers), current density, electrical resistance, and duration [46]. Electrical resistivity is an indicator of the composition and electrical conductivity of food [64].

OH differs from microwave and inductive heating methods in that it performs the heating process while in contact with food [65]. Compared to conventional heat treatment, it is effective in enzyme inactivation as well as guaranteeing the microbiological safety of the product [58]. OH, which has many advantages over fermentation and enzymatic hydrolysis, adjusts enzymatic activity and is seen as an important technique due to its positive effects on the properties of whey proteins (gelation, allergy). Electric field intensity affects the sensory and physicochemical properties of proteins and is also important in the production of bioactive peptides [66]. Alizadeh and Aliakbarlu [58] determined the antioxidant capacity of the fractions obtained by subjecting whey concentrate OH (50 Hz, 0–240 V, 5–15 seconds), UAE (24 kHz, 400 W, 25 mm titanium probe, 5–15 min), and enzymatic hydrolysis (pepsin). The degree of hydrolysis was increased by both pretreatments (excluding the combination of 5-min UAE and proteolysis), and the highest UAE was obtained with the treatment with a combination of 15 min, OH-15 seconds. It has been reported that this condition is caused by the breaking of covalent bonds and the denaturation of proteins by heat. It has been stated that antioxidant capacity increases depending on the degree of hydrolysis. In addition, UAE and OH showed similar effects when administered alone [58].

3.2.6 Subcritical water hydrolysis (SWE)

Subcritical water hydrolysis (SWE) (pressurized low-polarity compressed or pressurized hot water extraction) is a technology used for extraction or hydrolysis. In the method, it is possible to obtain liquid water with different polarities by decreasing the density and dielectric coefficient of water with the increase in pressure (0.10–22.1 MPa) and temperature (100–374°C) [67, 68]. With the increase of hydroxide and hydronium ions formed as a result of temperature and intermolecular vibrations, water behaves as an acid and/or base, becoming suitable for hydrolysis reactions [69]. The effectiveness of SWE depends on factors such as particle size, temperature, extraction time, flow rate, pressure, and solvent/sample ratio [67]. In addition, the type of atmosphere, raw material content, and reactor type can affect hydrolysis [70].

Unlike UAE, MAE, and HHP, which were applied before conventional hydrolysis methods, it is also effective alone and is a new alternative that has been successfully used in many substrates. In addition, SWE with additives (nitrogen, carbon dioxide, acetic acid, formic acid, sodium hydroxide, hydrochloric acid, and sodium chloride) can increase the yield and reduce the time and energy required. Although there are not many studies on whey SWE, promising results have been reported [46]. It has been reported that sodium bicarbonate increases the hydrolysis in SWE

(291°C, 28 min) with five catalysts in whey and a low molecular weight peptide is obtained [47]. Briefly, with the optimization of the concentration, temperature, time, and pressure of the additive (acetic acid, lactic acid, etc.) used in SWE, the degree of hydrolysis of proteins increases and low molecular weight peptides are obtained [46].

4. Future perspective

The health-beneficial components of many foods are attracting attention day by day, and the methods used to isolate these components are gaining importance. However, factors that will affect the properties of individual bioactive peptides to be obtained by separating whey proteins into their fractions make it necessary to develop the method to be used. In addition, while whey proteins are used for many purposes in the industry, it may be possible to produce more valuable bioactive peptides industrially with appropriate methods. Activation and isolation of bioactive peptides by conventional methods have been shown in many studies. However, it has been shown by a limited number of studies that it is possible to obtain more and different peptides before the application of novel methods such as UAE and/or HHP before conventional methods. The synergistic effects of new and traditional methods also need to be demonstrated. In addition, there are very few studies on whey proteins of SWE and OH methods, which are used to obtain peptides in various protein sources. It is important to produce novel, different, safe, and functional peptides using faster and lower-cost methods. In the future, there is also a need for studies on the optimization of these novel technology conditions. In addition, after the application of new methods applied as pretreatment, the activities of peptides need to be investigated further.

5. Conclusions

Whey is a waste and/or by-product that is important to utilize because of both its high oxygen demand and its rich composition. Whey contains proteins of high biological value, including essential and branched-chain amino acids, and is a source of bioactive peptides. Whey peptides individually have better bioactivity than whey proteins. While bioactive peptides are obtained, their physiological properties vary depending on the protein composition, processing conditions (such as pH, and temperature), and the method used. However, existing methods such as enzymatic or fermentation and novel technologies such as USE, HHP, and MAE, which are not very effective when used alone, are used together to obtain better peptides with efficient, nutritional, and biological properties. In addition to being environmentally friendly and economical, novel methods also have positive effects such as reducing the amount of enzyme in enzyme hydrolysis and shortening the fermentation period. The novel methods applied as pretreatment make the bonds and structures more sensitive by unfolding/denaturation of proteins with effects such as surface hydrophobicity and sulfide bonds. Thus, it is possible to obtain peptides with lower molecular weight in the second step. In addition, SWE and OH methods applied alone can yield peptides with low molecular weight, but further studies with whey proteins are needed. In addition to the extraction method to be used in the isolation

of whey peptides for industrial use, fractionation and purification methods are also effective in the activities of peptides. There is a need for the development of peptide production on an industrial scale.

Conflict of interest

The authors declare no conflict of interest.

Author details

Tugba Kilic
Kilis 7 Aralik University, Kilis, Turkey

*Address all correspondence to: tubacelik@kilis.edu.tr

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Chapter 2

Bovine Lactoferrin: Physiological Importance, Extraction and Application

Marta Liliane de Vasconcelos, Sonja Kukuljan and Todor Vasiljevic

Abstract

Lactoferrin (LF) is one of the minor milk proteins that has been gaining attention for its beneficial physiological functions to human health, as an antioxidant, antimicrobial, anti-inflammatory, immunomodulatory, anti-viral and bone growth agent. These characteristics are partly attributed to its ability to bind iron, which results in regulating the sequestration and release of iron in the body and partly due to its ability to interact with the molecular and cellular components of pathogens and their hosts. For this reason, LF is marketed as a functional component in various types of products, including infant formula, and in tablets or other types of supplements for children and adults. Bovine LF (BLF) is the main commercial protein ingredient from large-scale extraction using various technologies. The use of cationic exchange chromatography is the most common technology, and allows BLF to be extracted with around 95% purity and 87–93% of the isolated protein. Due to its characteristics and its various health benefits, BLF has also been studied as an additional component in new therapeutic applications in serious diseases such as upper and lower respiratory tract infections and COVID-19, cancers, for use in dermatology and regenerative medicinal engineering.

Keywords: bovine whey, ion exchange chromatography, iron transport, antimicrobial, transferrin

1. Introduction

The proteins of milk have great importance in human nutrition as they provide essential amino acids and amino groups for the biosynthesis of nonessential amino acids required for body development. Bovine milk typically contains 3.5–4% proteins, which varies considerably due to a number of factors [1]. Almost 80% of these proteins consist of caseins, and the remaining 20% of total milk protein is comprised whey proteins (WPs), predominantly consisting of α-lactalbumin (α-La) and β-lactoglobulin (β-Lg), and minor proteins including immunoglobulins (Ig), lactoferrin (LF) and lactoperoxidase (LP) [2]. Whey proteins and peptides derived from them exert multiple biological activities and functions including antioxidative,

anticancer, antimicrobial, anti-inflammatory, and immunomodulatory [3, 4]. Among minor whey proteins, bovine lactoferrin (BLF) has gained increasing attention due to experimental evidence suggesting that isolated LF expresses multiple health-promoting activities in human physiology improving iron transport, antimicrobial activity and immune responses [5].

Lactoferrin is a multifunctional protein, a non-hemic iron-binding monomeric glycoprotein, classified as a member of the transferrin family such as serum transferrin, melanotransferrin and ovotransferrin [5, 6]. It is present at varying levels in mammals (e.g. human, bovine, ovine, camel, caprine, elephant, alpaca), especially in the milk and colostrum of human and bovine origin and in lower concentrations in other biological secretions including saliva, tears, seminal and vaginal fluids [3, 5–8]. However, the LF is mainly commercially extracted from bovine milk, and it is subsequently added to many commercial products such as nutritional supplements, infant formula and cosmetics [6]. LF, also known as lactotransferrin, represents 1% of total whey proteins and was first identified in 1939 and denominated as a red protein in whey and isolated later, in 1960, from both human and bovine milk [4, 9].

Due to its importance in human health and the commercial interests in regard to BLF properties, this chapter outlines the main characteristics of this protein and how it impacts human physiology. In addition, approaches to its extraction and applications are highlighted including its behavior under various extraction and processing conditions. The current state of knowledge in terms of the protein's physiological functionalities is provided with the main point of emphasis.

2. Molecular characteristics of lactoferrin

2.1 Structure and concentration

The milk of many species contains iron-binding proteins including LF and lactoperoxidase. Both proteins share some common characteristics, such as molecular mass of around 80 kDa and iron-binding capacity [8, 10]. However, LF has affinity constant for iron 300 times greater than that of transferrin, and it can retain iron at a lower pH in part because of its basic nature [8].

The structure of BLF includes a tertiary polypeptide chain folded into two symmetric globular lobes (N-lobe and C-lobe), which are highly homologous with each other (between 33 and 41% homology) and joined by short α-helix (12 residues long from 333 to 344), that confers flexibility to the molecule and each lobe binding one Fe^{3+} [8, 11]. Its structure contains two spherical lobes with α/β domains, each of which can be subdivided into two sub-structural domains, namely the N1 and N2; C1 and C2 regions [12]. These four subdomains of LF are formatted as N1 composed of residues 1-90 and 251–333; N2 composed of residues 91-250; C1 composed of 345-431 and 593-676 residues; and the C2 sub lobe of 432–592 residues [6].

Within each LF lobe there are two Fe^{3+} ions, along with two CO_3^{2-} ions; the synergistic relationship between the ferric ion bond is an important feature that brings additional benefits to human health [11]. The iron-binding site is located in each lobe and the iron atoms are coordinated by four amino acid ligands: two tyrosine residues, one histidine and one aspartate. In the N-lobe, the iron ligands are Tyr 92, Tyr 192, His 253 and Asp 60, and in the C-lobe, Asp 395, Tyr 433, Tyr 526, His 595 and the ion CO_3^{2-}—in the binding site also to a synergistic anion—normally carbonate [8, 11]. The carbonate ion occupies a positively charged pocket in the

wall of domain 2 (N-2 or C-2), with this pocket being formed by the N terminus of helix α5 and the side-chains of an arginine residue (Arg121 in the N-lobe, Arg463 in the C-lobe) and a threonine residue (Thr117 and Thr459). This results in a set of hydrogen bonds that satisfy the full hydrogen bonding potential of the carbonate ion, and allow it to fit perfectly between the iron atom and anion binding pocket [11]. Interestingly, as observed by Moore et al. [11], the binding cleft N-lobe demonstrates a more open conformation in comparison to the C-lobe, although these differences do not correlate with iron affinity.

LF concentration in human milk ranges from 2.03 to 5.79 mg/mL [13], while in bovine milk it is 0.02–0.35 mg/mL. Substantial changes in LF content are usually observed during the transition from colostrum to milk since these concentrations are around 1.0–3.2 mg/mL (**Table 1**) [5]. These differences are influenced by the parity of the cow, stage of lactation and the somatic cell count [16]. Hiss et al. [14] demonstrated that the changes in LF concentrations during the colostrum phase and towards the end of lactation are due to physiological alterations, rather than innate pathogen responses. In colostrum, LF plays a protective role for the neonate, on the other hand, during the last weeks of lactation it protects the mammary gland.

2.2 Forms of lactoferrin based on iron saturation

Due to LF's reversible iron saturation characteristic, it is possible to find LF commercially in three forms: as holo-LF bound with two ferric ions and the saturation level between 76 and 100%; apo-LF (saturation level < 5%) one iron or iron-depleted; and native-LF composed of a mixture of both forms accounting for approximately 15–20% of metal saturation (**Figure 1**) [12, 17].

The presence of iron also determines the LF discoloration level, as the color transition to red occurs gradually with increasing iron saturation. When iron saturation is lower, the solution of LF is pinkish-to-off white and gradually reduces to white as iron saturation is reduced further [12]. Iron saturation is a crucial factor that affects the structure and thermal stability of LF. Holo-LF is denser than apo-LF, due to the binding of LFs with Fe3+, with a more folded structure, making it more resistant to heat and protein hydrolysis.

2.3 Surface properties of lactoferrin

Proteins carry a net negative or positive charge when the solution pH is below or above their isoelectric point (pI) [4]. LF is a positively charged protein at a physiological pH with an isoelectric point of approximately 8.0–8.5, which may be due to

Species	Concentration (mg/mL)	References
Bovine milk	0.02–0.35	[5]
Bovine colostrum	1.0–3.2	[5]
Human milk	2.03–5.79	[13]
Caprine milk	0.02–0.1	[14]
Ovine milk	0.06–0.1	[15]

Table 1.
Lactoferrin concentration in bovine, human, caprine and ovine milk.

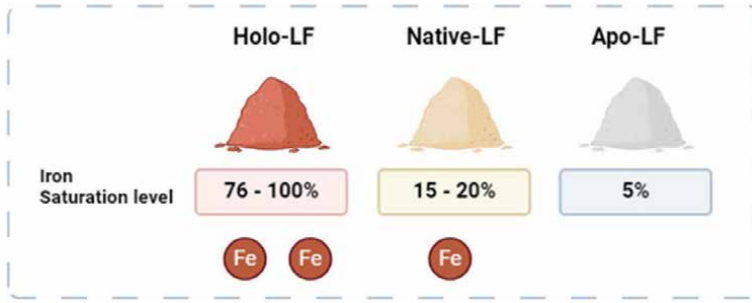

Figure 1.
Different levels of LF iron saturation.

a unique basic region at the N terminus [12, 13]. Lactoperoxidase, which also has an iron-binding capacity, has a similar pI of around 9.0 [18]. Changes in pH can affect the surface net charge of the proteins. Generally, at extreme pH, the protein molecules tend to unfold due to the exposure of buried functional groups. At intermediate pH, the ionization of side chains from the protein molecules will alter hydrogen bonding and salt bridges, which could destabilize the structure of the protein [19].

Cow, buffalo, goat and sheep LF share over 90% of amino acid sequence identity with each other [8]. The BLF contains 689 amino acids, highly conserved in internal regions, reflecting a stable and folded structure. On the other hand, amino acid substitutions occur readily on the protein surface. In this case, its surface features show some unique characteristics, such as charge, glycosylation and the generation of adventitious binding sites for other proteins, nucleic acids, small molecules or ions, arising from local combinations of amino acid substitutions [13, 20]. The major site is a spot of positive charge, which is located on the N1-lobe in the bottom left corner, encompassing the poly-peptide N-terminus. The cation region is also found in the inter-lobe region, associated with the helix that connects the two lobes. The major basic region surrounding the N2-terminus has been shown to be responsible for the binding of DNA, heparin, lipopolysaccharide and glycosaminoglycans by LF [20].

Bovine LF is a basic glycoprotein with 101 positively charged amino acid residues and 76 negatively charged amino acid residues. In a solution system with neutral pH, BLF is positively charged [19]. All LFs have the same fold, irrespective of the number or the location of their bound glycan chains. Most of the glycosylation sites are highly exposed on the protein surface, and the sugar residues have minimal interaction with the protein structure, at most a few hydrogen bonds. In particular, bovine LF has five sites on residues (Asn 233, 281, 368, 476 and 545), but only four sites are usually glycosylated in bovine LF, on Asn233, Asn368, Asn476 and Asn545 [21]. The sugars found on bovine LF include monosaccharides mannose, galactose, N-acetlyglucosamine, fucose, sialic acid and N-glycolylneuraminic acid [22].

Hydrophobicity is a phenomenon where the hydrophobic side chains of native proteins clump together due to their repulsion against water molecules. The protein surface hydrophobicity is related to the distribution of hydrophobic amino acid residues (e.g., Phe, Trp, Leu, Ala) on the surface of the protein molecules. Therefore, the hydrophobic sites exposed on the LF surface can be used to characterize the differences in protein conformation. The binding of ferric iron to transferrin could influence conformational changes (exposure of hydrophobic groups). In this case, protein molecules bind to each other through hydrophobic forces and protein aggregation occurs [19].

3. Nutritional importance of lactoferrin

Whey proteins are highly valuable as they are the source of all essential amino acids, and as such play an integral part in human health [4]. BLF, especially, plays an important role in many physiological pathways. It is used as a supplement to help promote iron absorption in the treatment of anemia in children, pregnant women and adults, as an antimicrobial agent and for bone growth. LF also inhibits the formation of ROS by binding to iron and thus attenuating oxidation (**Figure 2**) [23–25].

3.1 Iron delivery

The fundamental role of LF is to control the delivery of Fe3+, a required mineral cofactor for essential enzymes involved in many basic cellular functions and metabolic pathways. As a consequence, iron deficiency causes fatigue and decreased immunity [6, 26]. The oral administration of BLF through infant formula is also considered important for the provision and transport of iron during rapid postnatal growth, given iron insufficiency and deficiency in young children are among the most significant nutritional deficiencies globally [23, 24, 27].

Anemia and iron deficiency are also common in women during pregnancy and lactation [24]. Bovine LF is capable of restoring the physiological transport of iron from tissues to circulation, thus it can be used as a safe and effective treatment for pregnant women [23, 28]. Anemia affects approximately 30% women of reproductive age, 37% of pregnant women, and 40% of children 6–59 months age [29]. As a transporter of iron in iron recycling, LF can facilitate iron balance within the normal range and help avoid both iron deficiency and iron overload. LF significantly increases hematological parameters including hemoglobin, mean corpuscular volume, serum iron, transferrin saturation and serum ferritin [4, 24]. Additionally, LF binds iron with higher affinity than transferrin [30].

The role of BLF in regulating iron homeostasis also appears to be an important mediator of BLF's anti-inflammatory effect. The proinflammatory cytokine IL-6 is

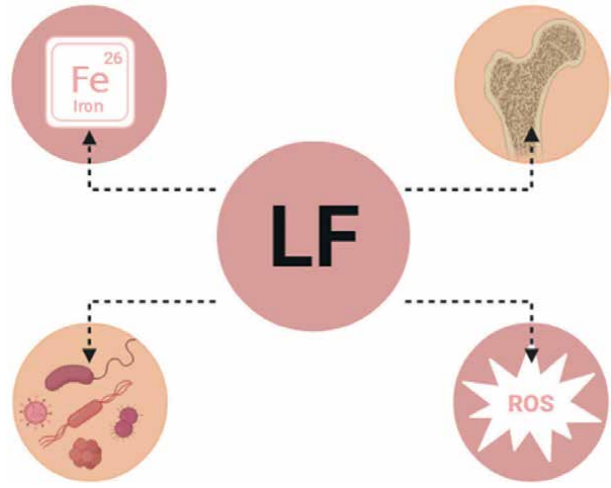

Figure 2.
Main physiological importance of LF in human health, including iron delivery, anti-pathogenic action, protection against reactive oxygen species (ROS) and also as a bone growth promoter.

reduced following BLF supplementation [31]. Ferroportin, which transports iron from tissues to the systemic circulation, is downregulated by IL-6. Conversely, hepcidin, which inhibits iron transport, is upregulated by IL-6 [32]. BLF treatment restores iron homeostasis and also reduces inflammation by lowering IL-6 and hepcidin and upregulating ferroportin [33, 34].

Briefly, the iron transport and storage system in the body starts with its absorption in the gut by enterocytes of the duodenum and jejunum, where part of the iron is absorbed while some is excreted, both in the Fe^{2+} and Fe^{3+} forms. Once absorbed, it follows the blood pathway by being contained in the hemoglobin of erythrocyte circulation, and then it is possible to be delivered and stored by other tissues such as muscle, as well as macrophages, enzymes and cytochromes. Thus, iron is available in the body to be used in metabolism. Post absorption from foods, especially Fe^{3+} is converted to Fe^{2+} then it can be stored and carried in enterocytes by iron-binding ferritin protein (Ftn) or released into circulation via basolateral membrane ferroportin-transporter (FPN). In circulation, Fe^{3+} ions bind to transferrin (TF) that transports it to all tissues [35].

3.2 Anti-inflammatory activity

LF shows potent anti-inflammatory actions, especially by affecting the expression of cytokine genes and regulating host immunity, lowering the levels of some proinflammatory cytokines such as IL-6, which, at high levels, lead to iron homeostasis disorders and tissue injuries [36]. LF demonstrates its anti-inflammatory properties at least partly by the transcription of cytokine genes (e.g. interferon-gamma (INF-γ), tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β) of the innate immune system. This multifunctional protein is highly resistant to GI digestion and following its intact absorption into the bloodstream, has the potential to regulate several intracellular signaling pathways [3]. LF also efficiently binds to specific membrane receptors of various tissues but especially of the immune system, including monocytes, lymphocytes, adipocytes, hepatocytes, enterocytes, and endothelial cells, in order to exert its physiological activities.

Inflammation is a process that involves many mediators and different types of cells in response to external factors (i.e. bacterial or chemical agents). LF is an immune mediator during the development of innate responses to injury and infection, insult and induced reactions of immune homeostasis, including mediation of cytokines, chemokines and specific cell surface receptors. The production of immunologic mediators that include cytokines and chemokines, depends on the recruitment of inflammatory cells of the innate immune system, such as neutrophils, macrophages and DCs, leading to the release of secondary mediators and subsequently induction of adaptive responses. There are two forms in which LF is found: the first form is contained in the secretions of the exocrine glands and the second is secreted from within secondary neutrophil granules. The LF of neutrophils has immunomodulatory functions in infections induced by modular responses of the monocyte/macrophage system, that produce inflammatory mediators, which in turn induce bone marrow to generate new immune cells and activate neutrophil degranulation. Subsequently, an amount of LF is released from the neutrophils to combat infections. LF interacts with innate receptors, such as Toll-like receptors 4 and 2, CD14, CD22 and CD40, which mediate inflammatory activities. It affects the activation of the 'danger signal' of monocytes/macrophages, competes with bacterium for receptor binding and can attenuate NF-KB, which induces the transcription of genes for various inflammatory

mediators like IL-6, IL-8, IL-12, IL-15, TNF-α, IFN-α and β, IL-1α and β. These mediators initiate a response cascade that includes bone marrow stimulation to produce monocytes and neutrophils. Therefore, LF is mechanistically acting as a feedback mediator for acute inflammation [35, 37].

3.3 Antibacterial activity

LF is described as a pleiotropic protein with antioxidant, anti-inflammatory, antibacterial, antiviral, antifungal, anticancer and immunomodulatory actions [3]. One of the most well-known roles of LF is its antibacterial function. Several *in vitro* and *in vivo* studies have demonstrated LF's ability to protect against microbial infections. It is well documented that LF exhibits an inhibitory effect against several Gram-positive and Gram-negative species, including some strains that are antibiotic resistant [38]. Therefore, LF has potential to become an adjunctive antibiotic therapy [39].

The mechanisms through which LF exerts its therapeutic effects are both bacteriostatic and bactericidal in nature [38]. In addition to the mechanisms, LF demonstrates in destroying Gram-negative and Gram-positive bacteria, LF also has the ability to prevent the attachment of bacteria to host cells, and also efficiently inhibits the growth of common pathogenic bacteria such as *Escherichia coli*, *Klebsiella pneumoniae* and *Listeria monocytogenes* [38, 40].

Bacteria use iron in DNA and RNA synthesis and as a source of energy [41]. One way by which the bacteriostatic effect of LF is mediated is by its ability to bind the Fe3+ ion, an action performed most effectively by apo-LF, which sequesters extracellular bioavailable iron, and which consequently deprives the use of this nutrient by bacteria at the site of infection. This iron sequestering effect might also inhibit bacterial growth systemically, by reducing environmental iron availability and depleting the iron source required by pathogenic bacteria [30, 38, 42]. Even at extremely low LF concentrations, it acts as a microbial inhibitor against Gram-negative bacteria, such as *Escherichia coli*, *Cronobacter sakazakii* and *Salmonella typhimurium* [43].

Another mechanism by which LF mediates its bactericidal effect is by directly interacting with the bacterial surface. Specifically, LF damages the external membrane of the Gram-negative bacteria by interacting with the lipopolysaccharide fraction and increasing the bacterial membrane permeability [38]. The LF mechanism of action against Gram-positive bacteria involves its binding to the N-lobe, due to its high positive charge, to anionic molecules on the bacterial surface that could prevent the interaction between the lipopolysaccharides and the cations required for bacterial growth [6, 38, 44]. LF binds the anionic molecules (e.g. lipoteichoic acid) on the cell surface of Gram-positive bacteria. This electrostatic binding reduces the overall negative charge of the cell wall and facilitates the effectiveness of the antibacterial compounds [6].

3.4 Antiviral activity

LF has strong antiviral activity against a broad spectrum of naked and DNA and RNA viruses [45]. The ability of LF to chelate two ferric ions and surface structures could explain its antiviral activity by blocking cellular receptors and binding viral molecules [36, 45]. Therefore, it inhibits viral replication and the formation of Reactive Oxygen Species (ROS) viral by-products [36]. Recent studies have demonstrated the LF N-lobe and C-lobe function of attaching to the S proteins of SARS-CoV-2, thereby blocking interaction with ACE-2 receptors and preventing

host cell entry [46]. Specifically, the C-lobe of LF interacts with the receptors of the SARS-CoV-2 binding domain via electrostatic attraction [46]. Thus, LF could also be a promising tool for anti-viral activity, exercising protective and neutralizing effects against COVID-19-causing viruses.

3.5 Antioxidant activity

The ability of iron to donate or accept electrons makes LF an important cellular redox compound. However, iron supplementation needs to be controlled, because the ferrous iron in high concentrations is potentially toxic to cells, as it donates electrons to oxygen, thus causing the generation of free radicals [27, 30]. In contrast, LF inhibits the formation of ROS by binding to iron and thus attenuating oxidation, reduction processes and the generation of superoxide (O_2•), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•), collectively termed ROS [23]. Bovine LF has been used as a safe and non-toxic source of iron supplementation [27, 47]. All three forms of BLF protect the organism against ROS. However, native BLF has demonstrated greater protection capacity on DNA damage after being exposed to ROS than other forms of LF, suggesting that LF might suppress oxidative DNA damage by scavenging ROS and sequestering free iron. On the other hand, the antioxidant capacity of holo BLF appears independent of its iron sequester activity [48].

3.6 Bone growth

Lactoferrin administered orally also promotes bone growth by stimulating the proliferation and differentiation of osteoblasts and inhibiting their apoptosis [34]. Treatment with BLF accelerates bone formation by stimulating bone mineral deposition and accelerating bone growth in the initial stage of osteogenesis in an animal model [42]. The local application of BLF is effective in bone regeneration, likely mediated by its antimicrobial, anti-inflammatory and immunomodulatory functions, making this glycoprotein an attractive growth factor for bone regeneration [42, 49]. In fact, its application has also shown positive effects in attenuating bone mineral density decline in humans with osteoporosis. BLF supplementation improves bone formation markers and reduces resorption markers [49]. In addition, BLF treatment in osteoporosis has also been shown to improve bone architecture, as shown by micro-computed tomography [50]. Further, *in vitro* BLF is associated with greater expression of the vitamin D receptor, which is strongly related to greater vitamin D absorption. Adequate vitamin D status is crucial to calcium absorption, homeostasis and bone metabolism [51].

4. Technological characteristics and innovations

4.1 Stability of LF during processing and applications

Food products supplemented with LF are usually subjected to some form of processing, such as pasteurization or spray drying, in order to make them safe for human consumption and/or to extend their shelf life. LF is administered as a nutraceutical in tablets, and also as a functional component in several types of products, including infant formula, yogurt and dietary supplements for humans and pets [52, 53]. However, those treatments can have a deleterious effect on LF, especially

on the structure and biological functions. This fact has led to the development of non-thermal processes to replace the standard thermal treatments, such as high hydrostatic pressure, ultrasounds and irradiation or high-intensity pulsed electric fields. These technologies are considered an alternative to heat treatment as they have the ability to inactivate vegetative forms of pathogenic and spoilage microorganisms at lower temperatures than those used in conventional heat treatments [54]. However, they are not suitable for all types of products and regulatory approval is required by food safety enforcement agencies to use alternatives to thermal pasteurization.

The kinetic parameters for heat-induced denaturation of BLF depend on different levels of LF iron saturation, and to some extent glycosylation, and composition of the treatment media [55]. In one study [55], LF thermal stability and the loss of its immunoreactivity to specific antibodies after heating at different temperatures (from 72 to 85°C) and holding times were evaluated by DSC. Marked differences in the endotherms were observed for the apo and holo forms of LF. The endotherm of the holo form showed two peaks, with temperatures of maximum heat absorption at 74°C and 86°C, whereas the apo form showed a single peak with a maximum heat absorption at 61°C. The value of the denaturation enthalpy corresponding to the holo form was higher than that of the apo form, being 21.0 and 8.6 J/g, respectively. When measuring LF immunoreactivity after heat treatment, it was observed that the protein denatured more rapidly in its apo than holo form. LF was more thermo-stable in the phosphate buffer than in milk, regardless of the degree of saturation, which could be associated with pH decline during milk treatment and interactions with other milk proteins and/or milk minerals.

Bengoechea et al. [56] showed that the denaturation of BLF is characterized by two peaks of maximum heat absorption, one lobe denaturing at 60.4°C and the other at 89.1°C. Denaturation, defined as a change in the structural elements of proteins involving unfolding, refolding and aggregation, of LF is irreversible, progressing to the formation of small aggregates—nanoparticles, whose size depends on the heating rate and temperature of treatment. The rate of the aggregation and the size of particles formed during this process increased with the concomitant rise in the treatment temperature. The formed particles were stable over a wide range of pH and ionic strength of the aqueous phase.

The structural properties of BLF are influenced by the quality of the environment, mainly pH and ionic strength [57]. The native structure of BLF undergoes a change of conformation at acidic pH. At pH 2.0–3.0, BLF partly unfolds with a low content of α-helical structure (3%) compared to that present at physiological pH (around 20%), but still remains rich in β-structure (around 54% at both pH levels). The thermal stability of BLF is also impacted by pH levels, as a gradual reduction in the value of the maximum peak of denaturation is observed with a decrease in pH, from 70°C at pH 7.0 to 39°C at pH 3.0.

The iron-binding ability of LF is also affected by pH and ionic strength [58]. BLF appeared to be more stable when it was heat treated at acidic pH than at alkaline pH and in both cases, its stability was higher at lower ionic strengths. At pH 7.4 and 9.0, LF retained 75–84% of the iron bound after heat treatment at ionic strengths below 0.01 and the formation of aggregates was observed at an ionic strength of 0.1. However, at pH 3.5 and ionic strengths below 0.37, LF maintained the iron-bound levels, while precipitation was observed at 0.57 of ionic strength. In addition, 85% of the iron binding capacity was maintained after heat treatment at temperatures of 65°C for 30 min; 70°C for 15 min; 80°C for 10 min; and 90°C for 4 min at an ionic strength of 0.01.

On the other hand, Oria et al. [59] studied the influence of heat treatment on the activity of LF promoting the proliferation of a promonocytic cell line. The results showed that after treatment at 72°C for 20 s, 85°C for 20 min or 137°C for 8 s, there was not any difference in LF activity compared to that of the non-treated LF. Moreover, heat treatments lower than 85°C for 10 min, specifically those usually employed in milk pasteurization of 62.8°C for 30 min (LTLT, low-temperature long-time) or 72°C for 15 s (HTST, high-temperature short-time) had no effect on the antibacterial activity of BLF against the foodborne pathogens including *Escherichia coli* O157:H7, *Salmonella serovar enteritidis* and Listeria monocytogenes [60]. Heat treatment at 70°C for 10 min had no observable effect on the three forms of BLF (holo, native, iron-depleted) when it came to simulated digestion [61]. While it is usually expected to observe greater digestion of heat-treated samples during gastrointestinal digestion, this was not the case in this study. The iron release and iron binding ability of LF after heat treatment and digestion conditions were not affected by the iron content of both holo and native LF, which released their iron completely before reaching the intestinal digestion phase.

Alternative non-thermal processing has been introduced fairly recently with two main aims—provision of required safety and preservation of quality attributes of foods including highly valuable compounds such as immunoglobulins and BLF in milk. High-pressure processing is one of these non-thermal technologies in which the products are treated at pressures ranging between 100 and 800 MPa [62]. While HHP treatment can effectively eliminate vegetative bacterial forms, it also causes substantial structural modification of food proteins. It specifically influences the functional properties of proteins through the disruption and reorganization of hydrogen bonds and hydrophobic interactions and the separation of ion pairs [63].

In general, whey proteins are denatured, likely interacting with fragmented casein micelle resulting in various aggregated forms, substantially different from that observed with heat treatment [64]. The extent of BLF denaturation with HPP depends on processing parameters and the environment [65]. In one study, a degree of LF denaturation subjected to pressure treatment was estimated by measuring the loss of immunoreactivity. The samples were treated at pressures from 450 to 700 MPa for different holding times at 20°C to determine D values, which is defined as the time required for 90% protein denaturation at a constant pressure. The obtained D values were lower when LF was treated in whey than those in milk, and lower in both whey and milk than in phosphate buffer. For example, at 600 MPa, D values for LF denaturation were 992, 2068 and 12,048 s for the protein treated in whey, milk and buffer, respectively. These results indicate the importance of the treatment media for the stability of LF and also showed a direct relationship between pressure and time of treatment.

Franco et al. [66] examined the impact of high pressure at 400, 500 and 650 MPa for 15 min at 20°C on the BLF structure of different iron-saturation forms. The holo-LF appeared unaffected by the treatments, while apo-LF underwent conformational changes above 500 MPa inducing the loss of iron. The iron-binding site of the N-lobe in the apo form of lactoferrin is the first site to bind iron due to its more open conformation than the C-lobe, allowing easy access to iron [67]. It is also possible that more intense HPP changes the conformation of the iron-binding sites in the lobes of BLF and then affects its iron-holding capacity. BLF can also aggregate with other whey proteins through disulfide interchange when subjected to high-pressure treatment [68]. The iron-saturation level appears to govern antibacterial activity, with non-treated LF showing antibacterial activity (about 9 log cycles CFU/ml of

reduction) only in its apo and native forms in PBS [66]. This antibacterial activity was maintained up to and at 400 MPa, but it was completely lost after HPP at 500 or 650 MPa.

During the last decades, great effort has gone into improving infant formula manufacture to more closely resemble human milk. One of the main modifications applied to bovine milk is to adjust the proportion of whey to casein proteins. Recently, Bravo et al. [69] performed a study on the effect of very high hydrostatic pressure on the protein distribution in bovine skim milk. Several treatments at pressures from 250 to 900 MPa at 15°C were tested, and the proportion of whey: casein obtained after applying the highest pressures was very similar to that of human milk. However, the loss of minor proteins was observed, among them LF. The concentration of soluble LF decreased with pressure, particularly at treatments from 550 to 900 MPa for 5 min. This could limit the application of HPP for the preservation of such products, given the loss in nutrition can be significant.

4.2 Production of lactoferrin on a commercial scale

The Belgian Oleofina Company in 1985 was the first company to commercially extract BLF using a cation-exchange chromatography system [52]. They were followed by the MILEI GmbH in Germany which produced BLF from cheese whey or skim milk on a large scale starting in 1989 [52]. In 1997, Royal FrieslandCampina (The Netherlands) used SP Sepharose Big Beads resin which improved the processing of milk and related products into lactoferrin [70]. The general principles of the process are based on the properties of BLF and its susceptibility to various processing conditions. As outlined by Wakabayashi et al. [71], fresh raw skim milk or whey is passed through a cation-exchange column at native pH. Since some other proteins interact with the column (i.e. lactoperoxidase), it is first eluted with a low-ionic strength buffer. This is followed by a high-ionic strength elution which separates BLF from the column. The BLF-rich eluent is subsequently concentrated by ultrafiltration and the buffer is removed by infiltration. The last steps involve stabilization by application of low heat treatment, usually pasteurization and drying (freeze or spray drying) (**Figure 3**).

While there are approaches to produce recombinant human LF in transgenic animals, fungi, yeast, cell cultures or plants [72], isolation from mammalian fluids currently remains the only commercially feasible option presently, although this sector of LF production is advancing rapidly. The cell-culture-derived human LF [73] might be launched as a bio-similar compound in the near future [74] but scaling up these processes will be challenging. Bovine milk products have a long history in human nutrition, and BLF at a purity >95% has achieved 'Generally Recognized as Safe' (GRAS) status for ingestion [75].

The properties of BLF mean that it is best obtained in its native form. For this reason, raw skim milk appears the substrate of choice as it contains BLF in the most appropriate form. Denaturation and thus irreversible loss of iron and biological activity starts around 70°C at neutral pH and accelerates in more acidic surroundings [61]. The extent to which this impacts the BLF functionality remains unclear [76]. Since BLF is one of the whey proteins naturally present in the serum phase of milk, it is usually present as part of isolated whey—sweet and acid as a by-product of cheese, yogurt or casein production. While this by-stream was previously disposed of in the past, the industry evolved to understand the importance of separating and concentrating its valuable components including proteins, lactose and minerals by various

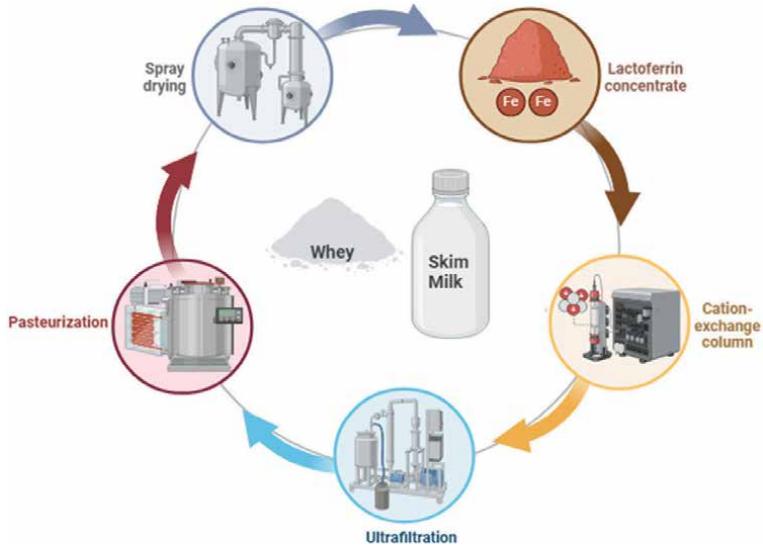

Figure 3.
Process to produce BLF concentrate using cation-exchange column, ultrafiltration, pasteurization and spray drying from whey protein or skim milk.

processes such as membrane and ion exchange systems. The levels of BLF in whey vary naturally, but this is also affected by the processing conditions prior to the solid concentration and whey separation [77], and the choice of the starting lactoferrin-containing media may be dictated by availability and necessity. However, newer entrants to Lactoferrin production are building their processing facilities to extract BLF from raw skim milk, which is becoming a preferred choice as it may contain up to 485 mg/L [16].

Most of the processes involving the extraction of BLF can be divided into several general steps, including fat removal, minimization of bacterial load by either bactofugation or membrane separation and some form of conditioning may be involved, such as pH adjustment. For example, Royal FrieslandCampina adjusts pH to 6.5-6.6 [70]. The chromatographic process starts with the preparation of the chosen chromatography resin, e.g., swelling and conditioning with a suitable aqueous salt solution, e.g., Royal FrieslandCampina uses a sodium phosphate buffer at pH 6.5 [70]. A feedstock is brought into contact with the chromatographic resin, where BLF and some other compounds adsorb to the cation-exchanger material. After completion of the adsorption step, unbound material is washed off with an equilibration buffer [70] or demineralized water [78]. BLF is usually eluted by a two steps elution process—firstly using a diluted sodium chloride solution, e.g., 1.6% (w/v) [70] which removes weakly bound proteins [78], followed by a high-salt solution, which may contain up to 10% sodium chloride, as in the MILEI or Synlait Milk Ltd. processes. After desorption, the BLF solution is desalted using an ultrafiltration process followed by diafiltration, membrane concentration, low-heat pasteurization and drying. The drying step can involve either freeze drying, such as in the case of MILEI, which obtains a powder with a protein content of 94.5% and a purity of 96% [79], or spray drying such as in the case of Synlait Milk Ltd., which results in a powder containing >95% of protein and a purity of >95% [78]. Some other isolated proteins can be further processed into whey protein concentrates or isolates [80] or be further fractionated into individual proteins [81].

Ion exchange separation appears to be the most feasible approach currently, as BLF is positively charged under most processing conditions, while most of other milk proteins carry a negative charge allowing for an easy separation. Most of the literature reports on strongly acidic cation exchangers with sulfonic acid-based functional groups such as sulfopropyl or methyl sulfonate, which perform well on a laboratory scale [82, 83]. The resin materials CM Sephadex C-50 [79], SP Sepharose Fast Flow [70] and SP Sepharose Big Beads [72, 80] are frequently used in the industrial processes. The SP Sepharose Big Beads, e.g., is a strong cation exchanger resin, designed for large-scale processes and suitable for viscous feedstock. The resin base matrix consists of large cross-linked agarose beads (100-300 mm) with sulfopropyl ligands coupled to the matrix via ether bonds [84].

While the use of ion exchange is likely the most commercially feasible option, some other approaches have been suggested. A completely different approach for BLF production involves membrane adsorption techniques where filtration and membrane adsorption were combined in laboratory and pilot scale studies for the isolation of BLF from sweet whey [82, 83]. For example, Ulber et al. [85] combined preconditioning of crude sweet cheese whey via crossflow microfiltration with selective BLF isolation via strong cation-exchanger membranes. BLF was eluted with a three-step sodium chloride buffer, and subsequently desalinated, concentrated and lyophilized, resulting in a BLF powder of about 95% purity based on protein [85]. Voswinkel et al. [82] proposed another approach that operated on a radial flow membrane adsorption chromatography system. At a pilot scale, a strong anion exchanger membrane and a strong cation exchanger membrane were cascaded in a customized liquid chromatography system. The system allowed for the separation of all minor proteins in milk since it alternated between two charged systems. Australian dairy company Murray Goulburn, now Saputo, proposed using a filtration process with a 50 kD cut-off membrane for the purification or enrichment of BLF from milk on a commercial scale [86]. Prior to filtration, the ionic strength of the feed is increased to more than an equivalent of 0.2 M sodium chloride or a conductivity of 20 mS/cm leading to selective enrichment of BLF.

A fairly new approach involving magnetic separation has attracted attention in recent years, especially for the isolation of minor whey proteins from whey combined with high-gradient magnetic separation (HGMS) [87]. In this approach, magnetic particles are added to the feed, where they selectively bind to the protein of choice. Subsequently, the protein-loaded magnetic particles are captured by a magnetized coarse filter matrix. The target protein is subsequently released from the magnetic particles into a suitable elution buffer and can be further processed into the final product. Comprehensive reviews of the applicability of whey [88] and the scalability of magnetic separation processes beyond the laboratory scale were recently published [87, 88]. Despite the recent availability of GMP-ready magnetic separation systems [89], and the potentially low operational costs [87], the technology has not yet been transferred to the commercial scale, primarily due to the lack of suitable commercially available magnetic particles for food production, especially at a moderate price and in large quantities [88].

5. Novel applications

The demand for LF has been increasing in recent decades due to greater awareness of its various beneficial functions. Bovine LF is used as an ingredient in infant

formula, particularly for the Chinese market and also in therapeutic goods and supplements [90]. Contemporary research on LF has focused on the discovery of new applications of BLF, new use cases, as well as applying BLF to improve existing treatment. **Figure 4** shows emerging new applications for BLF as well as different forms of use.

5.1 Use in dermatology

BLF has been also used topically to enhance skin care [91], as clinical trials report encouraging evidence that BLF may improve the treatment of some dermatological disorders, such as psoriasis, acne and oxidative change of the skin [38]. The skin is the largest and most external barrier of the body to the outer environment. It is richly perfused with immune cells and heavily colonized by microbial cells. Among them, there are a variety of microorganisms that are associated with skin conditions, such as particular *C. acnes* strains, abundance of *S. aureus*, *Saccharomyces cerevisiae*, *Demodex folliculorum* and *biofilm-forming bacteria* [92].

The fact that LF has potential effects on the microbiome plays an important role in a wide variety of disorders and is a key regulator for antifungal activity, anti-inflammatory properties and regulation of autoimmunity expression. Therefore, BLF and its various derived peptides in combination with other therapeutic methods may prove to be effective and safe in treating these [92, 93].

5.1.1 Psoriasis

Psoriasis is a chronic inflammatory skin condition and is classified as an immune-mediated inflammatory disease (IMID). It is characterized by red, scaly and thickened skin lesions that can occur at any site of the body [92, 94]. Three main categories of treatments are used in psoriasis: topical agents, phototherapy and systemic therapies [95]. LF may be included as a possible topical or oral treatment for psoriatic plaque [94, 95].

Antimicrobial peptides (AMP) are the key mechanism of innate immune responses, as a primary system of the epithelial barriers for protection in response to microbial invasion. AMP dysfunction is a central factor in the pathogenesis of psoriasis, that initiates the autoimmune-inflammatory cascade in psoriasis, stimulating pathogenic autoimmune T cells and then cytokines [94]. Moderate-to-severe psoriasis

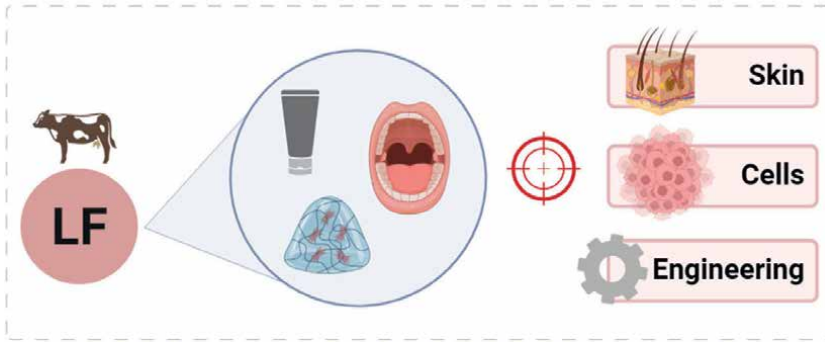

Figure 4.
BLF and its different forms for oral, topical and biomaterial compositions.

is treated with therapeutic antibodies that target these cytokines [92]. The mean microbiota shift associated with psoriasis is a higher abundance of *Staphylococcus* and *Streptococcus spp.* [92]. BLF may compete with AMP, down-regulate their expressions and the development of autoimmunity. In addition, LF may play its direct antimicrobial role by limiting the proliferation and adhesion [94].

5.1.2 Acnes vulgaris

Acne vulgaris is an inflammatory skin disease that is associated with an oily gland that often results in the production of sebum, altered keratinization, inflammation and bacterial colonization of follicles, mainly in regions of the face, back, neck and thorax. The first line, usual treatment for acne vulgaris is oral antibiotics combined with topical bactericidal medication [96]. Bovine LF may also be an adjunctive therapy in the treatment of acne vulgaris. Clinical studies using oral BLF as a treatment for moderate acne vulgaris have shown an improvement in injury count, skin status scores and change in acne severity scores compared to baseline after 8 weeks [97, 98].

5.1.3 Candida

Candida is a fungal infection and can become a serious skin problem. Several azole drugs are included in the treatment against *Candida spp*., but there is a well-known resistance of *Candida spp*. for these drugs, making them less effective [99]. When BLF is included together in the treatment, it has been shown that drug effectiveness against the fungus improves, via a synergistic action [93]. The mechanisms of action of BLF occur through direct and indirect interactions with several fungal pathogens that have been studied, including *Aspergillus fumigatus*, *Trichophyton mentagrophytes*, *Candida albicans* and *Candida krusei*. Similar to the effect on bacteria, the ability of BLF to sequester Fe3+, and alter membrane permeability are ways in which BLF can act as a fungistatic effect agent against *Candida spp*. [38]. In addition, LF is a source of peptides that are cleaved by various proteolytic enzymes. Among them, lactoferricin (bLfcin), lactoferrampin (Lfampin), and Lf (1–11) have been recognized for their antifungal activity [93, 100]. The synergy between peptides and azole antifungal drugs was observed to inhibit the growth of fungal, stimulate immunomodulatory properties and significantly reduce biofilm formation. However, the mechanistic basis of these synergistic actions remains unknown [93].

5.2 Anti-cancer applications

BLF appears to be a natural agent against cancer cells because there are indications that it leads to the cell cycle shutdown, and induction of apoptosis of these cells [100, 101]. Increasing evidence suggests that BLF is a safe agent capable of inducing anti-cancer effects in breast cancer [102–104]. The effect of apoptosis induction by BLF seems to be the main anti-cancer pathway since modifications in the expression of apoptotic genes have been observed, such as reduction of the anti-apoptotic gene Bcl2, increase of the pro-apoptotic gene Bid, and inhibition of growth in tumor cells, especially MDA-MB 231 cancer cell lines following incubation with BLF [102]. The same observations were noted when peptides derived from BLF were used, for example, the action of bovine lactoferricin (LfcinB) that showed induced apoptosis in different cells of cancer human *in vitro* [105].

In vitro study results also show the potential of BLF conjugated with iron oxide nanoparticles against the growth of gastric cancer cells. In this case, it is suggested that BLF interacts with proteoglycan, glycosaminoglycan and sialic acid, which are present on the surface of cancer cells exerting cytotoxic effects [101]. In addition, there is a study that showed that holo-BLF showed better anticancer properties compared to apo-BLF [104], possibly due to increased ROS due to decreased iron sequestration and increased labile iron. However, this possible difference between LF isoforms against cancer cells still requires further investigation [106].

6. Conclusion

BLF and its commercially available peptide derivatives have important roles in human health, with the main function of improving and supporting the biological functions of organisms (anti-inflammatory, immunomodulatory, anti-pathogenic, antioxidant and osseous growth). Current extraction methods are effective but still require adjustments to more efficiently achieve the required levels of protein purity, quantity and quality. Although its application is very well consolidated in improving the health of children, pregnant women, and in supporting the treatment of COVID, more research is still needed both *in vivo* and *in vitro* to explore greater potentials, as well as the best forms of application and dose-response. Moreover, there is potential to use BLF in the treatment of cancer, skin disease and it also shows promise in regenerative tissue engineering in bone medicine in combination with other biomaterials.

Author details

Marta Liliane de Vasconcelos[1*], Sonja Kukuljan[2] and Todor Vasiljevic[3]

1 Faculty of Animal Science and Food Engineering, University of São Paulo, Pirassununga, SP, Brazil

2 Noumi Limited, Ingleburn, NSW, Australia

3 Institute for Sustainable Industries and Liveable Cities, Victoria University, Werribee campus, Hoppers Lane, Werribee, VIC, Australia

*Address all correspondence to: martavasconcelos@usp.br

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Chapter 3

Hydrolysis of Lactose: Conventional Techniques and Enzyme Immobilization Strategies on Polymeric Supports

Jorge Lucas Vallejo-García, Alessandra Cutillo-Foraster, Ana Arnaiz, Saúl Vallejos, José M. García, María Asunción Muñoz Santamaría and Miriam Trigo-López

Abstract

This chapter explores lactose hydrolysis, emphasizing conventional techniques and the noteworthy immobilization of β-galactosidase on polymeric matrices to enhance the process. Lactose, present in milk and dairy, poses challenges for lactose-intolerant individuals, requiring enzymatic hydrolysis for lactose-free product development. The presence of other milk components, such as proteins and minerals, can indirectly influence the efficiency of lactose hydrolysis by potentially interacting with β-galactosidase enzyme or affecting its stability and activity, making it necessary to control factors such as enzyme concentration, temperature, pH, and reaction time to improve lactose hydrolysis rates. The chapter delves into established methodologies, covering enzymatic kinetics, reaction conditions, and substrate concentrations. It also describes the innovative approach of immobilizing β-galactosidase on polymeric supports to enhance enzyme stability, reusability, and overall efficiency in lactose hydrolysis. Discussions include the design of suitable polymeric matrices, providing insights into mechanisms governing catalytic performance. This comprehensive exploration contributes to understanding lactose hydrolysis, offering valuable insights for developing efficient and sustainable enzymatic processes applicable to the food and pharmaceutical industries.

Keywords: dairy products, lactose hydrolysis, enzyme immobilization, advanced polymers, polymeric supports

1. Introduction

The production and consumption of dairy products worldwide have increased in recent years, driven by factors such as population growth, rising incomes, and dietary preferences. Specifically, milk production is projected to reach 1020 million tons by 2030, making it the most consumed dairy product, followed by cheese [1].

These products are characterized by containing high amounts of lactose, or β-D-galactopyranosyl-(1 → 4)-D-glucose, which is a reducing disaccharide composed of the monosaccharides D-glucose and D-galactose linked by a β-(1 → 4)-glucosidic bond [2]. Lactose is naturally present in the milk produced in the mammary glands of mammals during the lactation period to provide energy to newborns. Lactose represents the predominant carbohydrate in milk, ranging from 4.4% to 5.2% in cow's milk and 6.5–7.5% in human milk [3]. The enzyme β-galactosidase, also known as lactase, found within the brush border microvilli of small intestine enterocytes in newborns [4], plays a crucial role in facilitating the hydrolysis of lactose, breaking it down into D-glucose and D-galactose. These resultant sugars are then absorbed, providing the essential energy required for growth and development (**Figure 1**) [2].

As previously stated, the global consumption of dairy products is substantial, leading to a significant lactose intake. Nonetheless, lactose intolerance issues prevent two-thirds of the population from consuming these products [5].

Lactose intolerance arises from the absence or deficiency of the enzyme β-galactosidase (hypolactasia) in the small intestine, impairing its digestion. Undigested lactose passes into the large intestine, where intestinal bacteria ferment the disaccharide. This fermentation process typically manifests within a short period, often between 30 minutes and 2 hours after consumption, leading to symptoms such as flatulence, bloating, abdominal cramps, or diarrhea [4, 5]. The prevalence and geographical distribution of lactose intolerance vary worldwide. For instance, over 90% of the population in Asia experiences lactose intolerance, while the figure is merely 2% in northern Europe. This disparity is attributed to genetic factors. Populations with historical and continued high consumption of dairy products, notably milk, harbor genetic mutations that sustain the enzymatic activity of intestinal β-galactosidase into adulthood, unlike other populations [4, 6].

β-Galactosidase deficiency can be classified into two main types: Primary, which is genetically determined, and secondary, which occurs because of diseases affecting the small intestine. Primary β-galactosidase deficiency includes congenital cases, present from birth and relatively rare, as well as β-galactosidase non-persistence, characterized by the natural decrease in the enzyme's activity after weaning. Conversely, secondary β-galactosidase deficiency is caused by underlying diseases that affect the small intestine [7]. The prevalence of β-galactosidase intolerance worldwide has spurred the development of industrial processes aimed at producing lactose-free products. These products are designed to be consumed by lactose-intolerant individuals, providing them with essential nutritional compounds found in dairy products without causing discomfort.

To qualify as "lactose-free," a product must contain a glucose-galactose disaccharide concentration below a specified threshold. This threshold is set for milk at 0.1 g/100 mL, as established by the European Food Safety Authority (EFSA) [7].

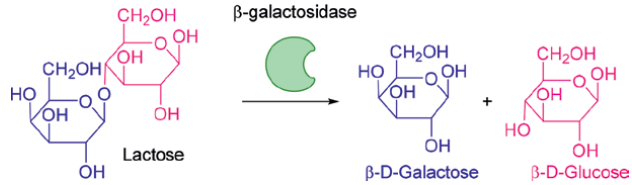


Figure 1.
The hydrolysis of lactose through the action of β-galactosidase.

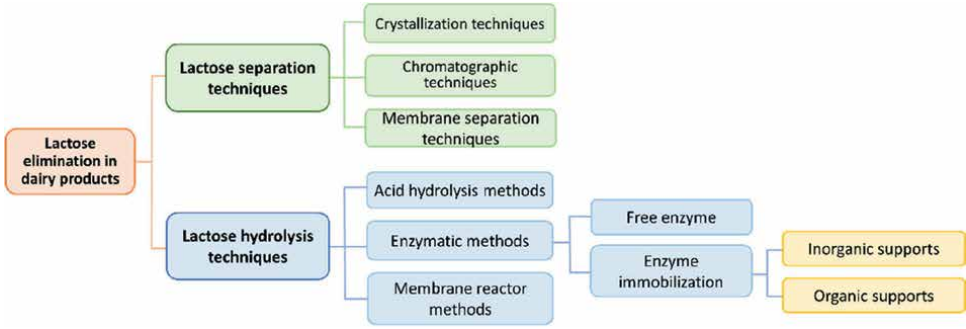


Figure 2.
Diagram of lactose removal techniques.

The removal of lactose from dairy products can be accomplished through separation or hydrolysis techniques. In separation methods, lactose is isolated from other milk components. Conversely, hydrolysis techniques involve the enzymatic or acidic breakdown of the β-(1 → 4)-glucosidic bond (**Figure 2**).

Industrially, most lactose-free products are produced through enzymatic hydrolysis techniques, where β-galactosidases are employed either as additives or coadjuvants. These enzymes are utilized either in free form, which can incur high costs and enzyme wastage, or in immobilized form, aiming to enhance enzyme stability and operational efficiency [8].

This chapter describes diverse techniques (illustrated in **Figure 2**) employed for lactose removal aimed at yielding lactose-free products. It offers a comprehensive exploration of separation and hydrolysis methodologies, elucidating their mechanisms and presenting instances from academic and industrial realms. Moreover, it conducts an in-depth description concerning the latest advancements in the immobilization of β-galactosidase onto polymeric supports for lactose elimination.

2. Lactose separation techniques

Lactose removal from a product can be achieved through separation techniques, where no enzymatic or chemical reactions are involved in breaking down this disaccharide; instead, it is simply isolated and removed. This lactose separation can be conducted through methods like crystallization, chromatography, and membrane separation. The resulting pure lactose obtained through these techniques finds application in the food industry as an ingredient in the production of various food items. Incorporating lactose into foods provides a range of unique functionalities, including low sweetness, reducing sugar properties, color/flavor carrying properties, protein stabilization, selective fermentation, or crystallization control, as well as physiological benefits such as prebiotic functions. It's widely used in various food products such as milk powder, infant formula, confectionery, baked goods, and as a base for dry food mixes [3].

2.1 Crystallization techniques

Crystallization is a physical process characterized by the formation of a crystalline solid resulting from cooling a saturated solution. Lactose exists in two anomeric

forms, α and β due to the existence of a chiral carbon. The most encountered form is a hydrated α-lactose monohydrate crystal that crystallizes at temperatures below 93.5°C [3, 9]. On an industrial scale, lactose crystallization is achieved through several sequential stages, including concentration, crystallization, and purification. Initially, dairy products undergo an evaporation process to eliminate water and concentrate lactose. The concentrated solution is subsequently transferred to a crystallization reactor, where lactose crystals gradually form via controlled cooling. Following crystallization, the crystals are separated from the solution using centrifugation. In the final purification stage, the crystals undergo washing and centrifugation again to remove impurities, yielding the desired final product. To optimize operational efficiency, it is crucial to maintain the temperature within specific ranges: Between 65°C and 70°C after the evaporation stage and between 25°C and 20°C during the crystallization process [9, 10]. It's important to understand that proteins can act as crystallization modifiers, since they have a significant impact on the formation and growth of crystals by affecting factors such as nucleation, crystal growth, and crystal habit. Studies suggest they diminish lactose solubility [11], expedite nucleation [12], and reduce lactose crystal size [13]. Although the precise mechanism of proteins' influence on lactose crystallization remains unclear, it's theorized that their water-binding capacity creates localized areas of lactose supersaturation [12]. Alternatively, some researchers suggest proteins may serve as nucleation centers, facilitating heterogeneous lactose nucleation [14, 15].

In industrial crystallization processes generally fine lactose crystals are obtained, while ideally the production of large and uniform crystals is essential to facilitate subsequent separation and handling. Preferably, lactose crystals should measure between 200 and 300 μm in size and exhibit a tomahawk shape [3, 15]. The size, distribution, and shape of crystals are contingent upon solution type and operational conditions. Additionally, the size and distribution of lactose seeds significantly impact nucleation and growth rates in crystallization. Therefore, in addition to conventional methods, negative temperature procedures to favor crystal growth [10], ultrasound to diminish induction times for nucleation and increase crystallization rate [16–18], or a flow of CO_2 and N_2 gases to increase the nucleation rate and yield [19] can also be employed during the crystallization stage to enhance crystal production. While the primary objective of this technique is not necessarily to produce lactose-free products, its application in removing lactose from dairy products can significantly contribute to the production of lactose-free products obtaining two products with added value in a single process. Understanding how varying percentages of lactose removal influence the production process can shed light on the scale and challenges associated with achieving lactose-free products using crystallization techniques. For instance, exploring how different levels of lactose removal affect the final taste, texture, and overall quality of the product provides valuable insights into the feasibility and optimization of producing lactose-free products [10].

An exemplary large-scale application of this technique is demonstrated by Fonterra, a dairy product company based in New Zealand. In 2016, Fonterra developed and patented an industrial crystallization process, primarily for lactose production, which indirectly facilitates the production of lactose-free dairy products. In this patented process, dairy products are initially heated to temperatures ranging between 50°C and 90°C in an evaporator to concentrate the lactose. Subsequently, the temperature is reduced to 8–25°C, promoting the formation of lactose crystals. These crystals are then separated from the rest of the solution through settling, followed by centrifugation and washing to eliminate impurities [20].

2.2 Chromatographic techniques

Chromatography is a versatile technique used to separate the various components of a mixture based on their differential retention while traversing through a supporting medium. This technique encompasses different variants depending on the characteristics of the stationary and mobile phases, as well as the interactions between the mixture components and these phases. Examples of chromatography types include ion-exchange chromatography (IEC), hydrophobic interaction chromatography (HIC), and affinity chromatography (AC), each one leveraging distinct interaction between the stationary phase and the mixture compounds for separation. Additionally, size-exclusion chromatography (SEC) represents another variant, wherein the separation is dictated by the size of the compounds [21].

Chromatographic techniques are not widely employed for lactose separation to produce lactose-free products on an industrial scale, primarily due to economic considerations. However, they find utility in determining lactose content and other compounds in dairy products. A notable exception is the application of chromatography to obtain lactose-free milk using a chromatographic column composed of poly(aniline) (PANI) functionalized with glutaraldehyde (GA) and lectin. This technique facilitated the removal of 47% of lactose from milk, leveraging lectin's high affinity for this disaccharide [22].

2.3 Membrane separation techniques

Membrane technologies rely on the application of pressure or electrostatic gradients to drive the passage of components in a solution through a semipermeable porous membrane. Components that are of a size compatible with the membrane pores and pass through are termed permeate, while those that are unable to permeate the membrane and are retained are referred to as retentate [21, 23].

In the dairy industry, membrane filtration techniques such as ultrafiltration (UF) and nanofiltration (NF) play a pivotal role in the recovery of various compounds from dairy products, including proteins, amino acids, and lactose. These methods operate on the principle of segregating particles based on their size using specialized membranes. UF membranes, with pore sizes ranging from 1 to 100 nm, facilitate the passage of small molecules like lactose, water, and minerals while retaining larger molecules such as proteins and fats. In contrast, NF membranes feature smaller pores (1–10 nm), enabling the retention of lactose and molecules of similar size while permitting smaller molecules like water and minerals to traverse through. Additionally, electro-dialysis (ED) techniques utilize electrically charged membranes to achieve separation via an electrostatic gradient, providing an alternative approach [23, 24]. Consequently, these techniques offer the potential to derive products with low lactose content or lactose-free products as byproducts of the filtration process.

The proper selection of membrane materials (ceramic or polymeric) is essential to ensure optimal separation efficiency. Polymeric membranes are more widely used for their affordability and lower energy demands compared to ceramic alternatives. Polymeric membrane materials used in production can be categorized into modified natural polymers, such as cellulose acetate, synthetic polymers including polysulfones, polytetrafluoroethylene, polyamides, polysulfides, and polypropylenes. Cellulose acetate membranes generally have lower thermal, chemical, and biological resistance compared to synthetic polymers, which offer

greater resilience to varying operational conditions such as temperature and pH. Conversely, ceramic membranes boast greater resilience owing to their physical, hydrothermal, and chemical stability properties. Depending on specific operational needs or applications, various materials such as Si, Zr, Ti, Al oxides, and silicon carbide (SiC) are utilized due to their distinct surface charge characteristics in solution. Ceramic membranes find common use in microfiltration (MF), UF, and NF applications. Additionally, they can be effectively cleansed using standard sanitizing products commonly employed in the food industry, thus enhancing their reusability (**Table 1**) [23, 25].

Considering the potential for obtaining lactose-free products through filtration and ED techniques, a study demonstrated the successful production of lactose-free powdered milk by integrating UF and NF techniques with ED [24]. Initially, the proteins and fats present in the dairy product are retained using a poly(ether sulfone) (PES) UF membrane. Subsequently, mineral salts are captured by the ED membrane to prevent fouling of the NF membrane and enhance the efficiency of lactose removal. It is noteworthy that the retained salts are reintroduced into the final lactose-free product at the conclusion of the process. Finally, lactose is retained in the NF membrane. This process achieved lactose-free powdered milk with a lactose content of less than 0.2%, along with lactose with a purity of 95.7%.

In addition to UF and NF membranes, MF membranes are utilized for their high permeation flow and efficiency in separating lactose and proteins in dairy products. A recent study [26] showcased the effectiveness of ceramic ZrO_2 and Al_2O_3 MF membranes in removing lactose from skim milk while simultaneously concentrating the protein in a single step. This resulted in 87.73% lactose removal and a product containing less than 5 g/L of lactose.

On an industrial scale, a Finnish dairy company (Valio) acquired a patent in 2010 for developing a process to produce milk with less than 0.01% lactose using membrane separation techniques. In an example of this process, 30 L of pasteurized milk with a 1.5% fat content undergo filtration at 50°C through a UF GR61PP membrane having a cut-off value of 20,000 Da, allowing lactose, mineral salts, and water to pass through while retaining proteins and fats. Subsequently, lactose is separated from the mineral salts and water using Millipore Nanomax-50 NF membrane, with lactose retained in the membrane and mineral salts and water forming the permeate. The mineral salts and water, comprising the NF permeate, are concentrated using a reverse osmosis membrane (Nanomax-95), where mineral salts are retained (NaCl retention > 94%) in the membrane while water passes through. Finally, the mineral salts recovered in the reverse osmosis stage (10.5 g) are mixed with the retained in the UF stage (69.2 g) and water (20.3 g). Then, 0.35 g of HA lactase is added and allowed to hydrolyze for 24 hours at 10°C resulting in lactose-free milk with identical organoleptic properties [27].

Membrane material	Optimum working pH	Optimum working temperature (°C)
Cellulose polyacetate	3–8	<50
Synthetic polymer	2–12	<80
Ceramic	0–14	>300

Table 1.
Optimal operational conditions according to the material of the membrane.

3. Lactose hydrolysis techniques

As highlighted earlier in this book chapter, various pathways have been devised for hydrolysis of lactose's β-(1 → 4)-glucosidic bond. These pathways encompass the chemical route, employing acid hydrolysis, and the biological route, employing enzymatic hydrolysis. Acid hydrolysis is facilitated by the presence of inorganic and organic acid compounds, while enzymatic hydrolysis utilizes biological catalysts such as enzymes. In addition to these two approaches, a third method known as membrane reactor method has emerged, which integrates enzymatic hydrolysis with separation stages. Below, we delve into the methodologies associated with acid hydrolysis, enzymatic hydrolysis, and membrane reactor techniques.

3.1 Acid hydrolysis methods

Acid hydrolysis of lactose involves the cleavage of the β-(1 → 4)-glucosidic bond through the action of acidic compounds like HCl, H_2SO_4, citric acid, and H_3PO_4 [28, 29]. In this process, the pH of the medium typically drops to around 1–2, and high-temperature conditions (100–150°C) are maintained [30]. While this method is commonly employed in whey or products obtained after milk UF, its aggressive conditions can result in protein degradation and the formation of undesired byproducts. Consequently, acid hydrolysis is not typically utilized for producing lactose-free products. However, it has found application in obtaining alternative products, such as lactose-free whey syrup with high glucose and galactose content, where hydrochloric acid combined with ultrasound reduces hydrolysis time to just 1 hour [31].

3.2 Enzymatic hydrolysis methods

The methods of enzymatic hydrolysis primarily involve the enzyme β-galactosidase, which, as mentioned, catalyzes the hydrolysis of the β-(1 → 4)-glucosidic bond present in galactosides. This tetrameric protein has an approximate molecular weight of 464 kDa, which may vary depending on its source. β-Galactosidase can be extracted from various biological sources, including bacteria, fungi, yeast, and plants. However, enzymes sourced from fungi such as *Aspergillus oryzae* and *Aspergillus niger*, as well as yeasts like *Kluyveromyces lactis* and *Kluyveromyces fragilis*, are the most commonly utilized in industrial applications [32]. The utilization of enzymes to produce lactose-free products can be accomplished by employing the enzyme in its free form or by immobilizing it on a support. Enzymatic lactose hydrolysis methods involve introducing the enzyme into a reactor containing a dairy product, facilitating the production of a lactose-free product through its catalytic action.

The optimal pH and temperature conditions for β-galactosidase, which exhibits maximal catalytic activity, are contingent upon its biological origin. Therefore, selecting the appropriate source of β-galactosidase is crucial to match the operational conditions required for effective hydrolysis. For instance, when hydrolyzing lactose in acid whey, β-galactosidases isolated from bacteria are preferred due to their optimal pH range between 2.5 and 5.4. Conversely, β-galactosidases sourced from yeasts are typically employed for lactose hydrolysis in milk and whey, as they demonstrate optimal pH activity within the range of 6.0–7.0 [32]. **Table 2** summarizes the key characteristics of β-galactosidases commonly utilized on an industrial scale.

Despite the availability of various enzymes sourced from different biological origins, efforts have been made to develop more efficient enzymes capable of

Biological origin of β-galactosidase	Microorganism type	Optimal temperature (°C)	Optimal pH
Aspergillus oryzae	Fungi	55–60	4.5–5.0
Aspergillus niger	Fungi	55	3.5–4.5
Kluyveromyces lactis	Yeast	30–35	6.5–7.0
Kluyveromyces fragilis	Yeast	30–35	6.0

Table 2.
Characteristics of the most common β-galactosidases employed in the dairy industry.

functioning across a broader pH range and at elevated temperatures (thermostable). Genetic engineering techniques have played a pivotal role in attaining this objective, enabling the genetic modification of organisms to facilitate the expression of proteins, such as tailored modified β-galactosidase, designed to fulfill precise specifications. These techniques involve the manipulation of genetic material to introduce, remove, or modify genes, thereby altering the genetic makeup of organisms to elicit desired traits or functions. In this context, the targeted modification of organisms to express a modified form of β-galactosidase underscores the versatility and precision afforded by genetic engineering in tailoring proteins to meet specific industrial, scientific, or therapeutic needs. β-Galactosidase has been subject to modification to enhance its stability, activity under particular conditions, or to confer specialized properties conducive to specific applications [33, 34].

3.2.1 Methods of enzymatic hydrolysis with free enzyme

In a study investigating the enzymatic hydrolysis of lactose in milk, cheese whey, and whey permeate ultrafiltrate using free β-galactosidases sourced from *A. oryzae* and *K. lactis*, researchers explored the effects of enzyme concentration, temperature, and reaction time depending on the biological origin of the enzyme [35]. At a temperature of 10°C, which is of particular interest for preserving nutritional and sensory composition while preventing microbial growth, regardless of concentration and reaction time, β-galactosidase from *K. lactis* demonstrated more efficient lactose hydrolysis in the mentioned dairy products compared to that from *A. oryzae*. However, complete hydrolysis of lactose, reaching 100%, was only achieved using *A. oryzae* at its optimal temperature of 55°C and after a reaction time of 12 hours.

On an industrial scale, lactose hydrolysis utilizing β-galactosidase has been widely employed to produce lactose-free dairy products such as milk, yogurt, cheese, and ice cream, among others, in two different processes, batch and aseptic. In the batch process, the enzyme is introduced into a reactor containing raw or thermized (15 seconds at 65°C) milk and allowed to incubate for 24 hours at temperatures between 4°C and 8°C to inhibit microbial growth. Then, the milk is pasteurized (this process also inactivates the enzyme), homogenized, and packaged. In the aseptic process, the first step in the milk sterilization following the ultra heat treatment (UHT) procedure (2 seconds at 142°C) and the sterile enzyme is then injected just before packaging. Because UHT milk is often stored in quarantine for around 3 days at room temperature, there is sufficient time for complete hydrolysis before the milk is sent to the retailer. Since pasteurized milk does not undergo a quarantine period, the aseptic process is not utilized for this type of lactose-free milk [36].

In most enzymatic lactose hydrolysis methods, β-galactosidase is typically the enzyme selected. However, as an alternative, β-glucosidase sourced from the archaea *Sulfolobus solfataricus* can be utilized. This enzyme exhibited broad substrate specificity across various oligosaccharides, encompassing lactose and glucose oligomers, alongside high thermostability and thermophilicity, withstanding temperatures of up to 80°C. Additionally, it demonstrated resilience against proteases, organic solvents, and detergents. Therefore, this other group of enzymes presents significant potential for industrial utilization due to their remarkable versatility and resilience [37].

3.2.2 Methods of enzyme immobilization

To mitigate the high operational costs associated with free enzymes, enzyme immobilization methods have emerged, involving the attachment of enzymes onto solid supports via covalent bonds or weak interactions. These methods address limitations such as the inability to reuse enzymes in multiple cycles and the susceptibility of enzymes to denaturation under working conditions, resulting in a rapid loss of catalytic activity. Immobilization reduces operational costs by facilitating enzyme reuse, enabling continuous operation, and enhancing enzymatic stability while also allowing for the separation of the enzyme from the product [38]. Achieving optimal stability and efficacy in the support-enzyme system requires careful selection of immobilization strategies to prevent conformational changes in the enzyme structure that could otherwise diminish its enzymatic activity and alter its kinetic properties [8].

Immobilization methods encompass both physical and chemical techniques. Physical methods include adsorption and entrapment, while chemical methods involve covalent binding and crosslinking (**Figure 3**). Adsorption immobilization entails attaching enzymes to support via non-covalent interactions like dipole-dipole interactions, ionic interactions, hydrophobic forces, van der Waals forces, and hydrogen bonds. This method is favored for its simplicity, requiring mild operational conditions of pH and temperature, supporting reuse potential due to the reversible bond between enzyme and support, and, crucially, preservation of enzyme structure, thereby maintaining catalytic activity. However, the weak interaction inherent in this immobilization type may lead to enzyme desorption from the support under varying operational conditions. Notably, ionic adsorption of enzymes on ion exchange resins is a notable subtype within adsorption immobilization [8, 39].

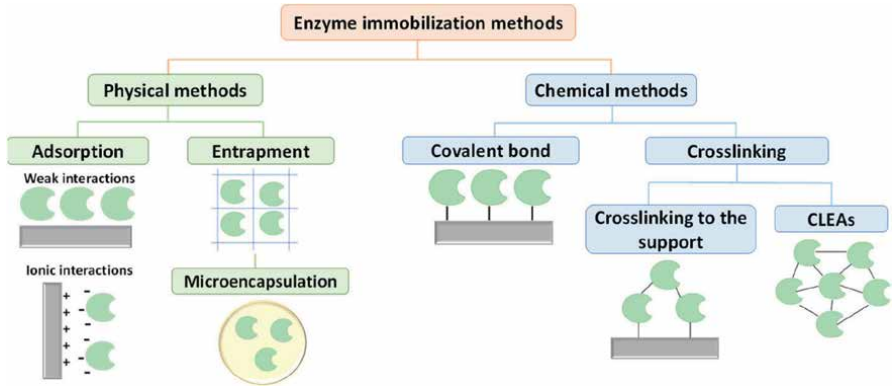


Figure 3.
Classification of different enzyme immobilization methods.

On the contrary, entrapment immobilization involves encapsulating the enzyme within a matrix. This physical method is known for its simplicity and ability to preserve the enzyme's structure, thus retaining enzymatic activity. However, the lack of strong interaction between the matrix and the enzyme may result in enzyme release under certain operational conditions. Moreover, diffusional limitations can impede substrate-enzyme active site contact or final product release [8, 40]. This method encompasses techniques such as microencapsulation, where the enzyme is enclosed within a semipermeable spherical membrane with controlled porosity [39].

In chemical methods, covalent binding immobilization involves the creation of covalent bonds between functional groups on the support's surface and amino acid residues on the enzyme's surface. This process can occur directly between the enzyme and the support or with the assistance of a bridging compound. Notably, this immobilization is typically irreversible. While the formation of strong bonds enhances the stability of the immobilized enzyme under varying operational conditions, it's important to acknowledge that this bond's nature may also induce structural changes in the enzyme, potentially impacting its catalytic activity and stability [41].

On the contrary, crosslinking immobilization utilizes a di- or multifunctional reagent such as GA, isocyanate, or *N*,*N*′-ethylenebismaleimide, with GA being the most prevalent, to form covalent bonds between enzyme molecules. Often, this method is coupled with other immobilization techniques to enhance system stability during operational conditions. Notably, crosslinking enables enzyme immobilization without any supports, known as crosslinked enzyme aggregates (CLEAs), by creating covalent bonds between enzymes [8, 40].

In addition to the various enzyme immobilization methods, it's crucial to underscore the significance of the different supports utilized for this purpose. A suitable support must fulfill several criteria, including stability under operational conditions, high surface area, biological inertness, resistance to biological degradation, cost-effectiveness, chemical and mechanical stability, and the presence of reactive groups on its surface [38]. Supports can be categorized as either inorganic or organic based on their nature. Inorganic supports are renowned for their exceptional mechanical, chemical, and electrical properties, along with their high surface area, porous structures, and cost-effectiveness due to their abundance. Among the frequently employed inorganic materials for immobilization are silica (SiO_2), metal oxides like ZnO, Al_2O_3, FeO, and Fe_2O_3, as well as minerals such as clay and bentonite [42]. Additionally, the growing utilization of nanomaterials, such as ZnO nanoparticles, is noteworthy due to their nanoscale dimensions, extensive contact surface area, resistance to mass transfer, and ability to enhance enzyme stability under varying operational conditions of temperature and pH [5].

Polymers are frequently utilized for enzyme immobilization when it comes to organic supports. Polymers are large molecules composed of monomers, which are repeated units along the chain. These supports are classified based on the origin of the polymer into natural, synthetic, and modified natural polymers. Natural polymers, or biopolymers, such as chitosan, agarose, and cellulose, are derived from living organisms and are recognized for their biocompatibility, biodegradability, low toxicity, and strong enzyme affinity [42]. In contrast, synthetic polymers such as polyethylene glycol (PEG) and poly(vinyl alcohol) (PVA) are synthetic and valued for their high versatility, ease of functionalization for specific applications, structural stability, and ability to shield biomolecules from degradation, thereby enhancing their stability [42, 43]. Hybrid or composite supports, consisting of a blend of inorganic and organic materials, offer a unique combination of properties derived from both components.

Commonly, these supports integrate nanomaterials with polymeric materials to leverage the advantages of both constituents [42, 44].

There is a growing interest in the development of effective supports for immobilizing β-galactosidase, given its significance in various industries. While carbon nanotubes and inorganic supports, particularly nanomaterials like nanodiamonds, ZnO nanoparticles, SiO_2 nanoparticles, and silver nanoparticles, have been utilized for this purpose due to their remarkable mechanical properties [45–51], the recent trend favors the use of polymeric supports. This preference is attributed to the abundance of polymeric materials, their versatility, and biocompatibility, offering promising prospects for β-galactosidase immobilization.

On an industrial scale, the Milan-based company Centrale de Latte (1975) pioneered the development of an industrial entrapment immobilization process for β-galactosidase, facilitating lactose-free milk production [52]. This process entails introducing milk into a reactor containing the enzyme immobilized in cellulose acetate fibers at a temperature of 5°C for 20 hours, resulting in a 75% lactose hydrolysis rate and enabling the production of 10,000 L of lactose-free milk per day. After each cycle, the fiber requires cleaning with a sterile buffer solution containing Mg^{2+} and treatment with a quaternary ammonium compound to prevent psychrophilic bacteria growth on the fiber's surface. Despite achieving significant operational stability, with only a 10% loss after the 50th cycle, this method does not operate continuously. The implementation of enzymatic immobilization on an industrial scale has paved the way for designing other immobilization systems for lactose-free product production. For instance, Corning company produced lactose-free whey by immobilizing β-galactosidase in porous glass beads. Valio, a Finnish company, also obtained lactose-free milk with the enzyme immobilized in a phenol-formaldehyde resin through adsorption and crosslinking with GA [52, 53].

3.3 Membrane reactor methods

In membrane reactors, a combination of UF membranes and enzymatic hydrolysis using β-galactosidase enables separation stages. The process hinges on the distinct interactions of lactose, the hydrolyzed product, and the enzyme with the UF membrane. In the general process, hydrolysis occurs in a protein-free milk or serum stream, purified by UF, where lactose is enriched in the permeate while the retentate has lower lactose content. The permeate then undergoes lactose hydrolysis in a reactor with β-galactosidase. The hydrolyzed product, passing through a second UF membrane, is mixed with the solution retained in the first UF stage to yield a lactose-free product. Meanwhile, the enzyme retained on the second UF membrane is recovered for another operational cycle [21, 54]. Despite the potential for continuous operation, microbial growth induced by non-sterile feedstock and operational complexities hinders widespread industrial adoption of this method, dampening commercial interest in membrane reactors [21].

In membrane reactor methods, the enzyme can either be immobilized on the UF membrane or in free form in the reactor. As an illustration of this, a work described a process where the lactose underwent hydrolysis using two hyperthermophilic β-galactosidases derived from archaea *S. solfataricus* and *Pyrococcus furiosus* [55]. This process occurred in a continuously stirred reactor integrated with a crossflow 10-kDa UF membrane, facilitating enzyme recovery. Throughout the operational period, which spanned over 2 weeks, the reactor maintained a consistent conversion rate of 80% while utilizing lactose as the substrate. In a separate instance, 43% of the lactose

present in skim milk underwent hydrolysis within a reactor featuring a PES UF membrane. In this setup, the enzyme was immobilized and crosslinked with 4% (v/v) GA [56]. Remarkably, the enzyme retained over 90% of its initial activity after 6 operational cycles. Likewise, lactose contained in cheese serum has been successfully hydrolyzed within a reactor housing β-galactosidase from *K. fragilis*. This enzyme was immobilized on a 10 kDa PES UF membrane activated with GA at a temperature of 55°C and a pH of 6.9 [57].

4. Enzyme immobilization on polymeric supports

The selection of the appropriate support for enzyme immobilization is paramount to ensure system efficiency. The material properties of the support significantly influence the enzyme's performance. Ideally, the support should exhibit high affinity with proteins, possess functional groups on its surface for enzyme interaction, demonstrate high mechanical stability, and be non-toxic, biocompatible, and biodegradable [58]. In addition, the presence of proteins or other components in milk can have complex effects on β-galactosidase activity in immobilized enzymes, which may require careful consideration and optimization in lactose hydrolysis processes.

Polymeric supports offer notable advantages over inorganic ones, particularly due to their versatility and exceptional mechanical properties, making them the preferred choice for enzyme and protein immobilization applications. As discussed previously, polymeric supports are categorized into two groups based on the polymer's origin: Natural and synthetic. Natural polymers, or biopolymers, are derived from living organisms and are specifically sourced from biologically derived polysaccharides or proteins. These polymers are esteemed for their biocompatibility, biodegradability, and non-toxicity. The natural origin of these polymers is responsible for their high biocompatibility with enzymes, which helps to minimize changes in the enzyme's structure and properties and achieve greater retention of enzymatic activity. Moreover, these materials are cost-effective in industrial settings as they originate from natural sources and can be readily obtained from nature or through industrial processes that generate them as byproducts. For all these reasons, polymeric supports based on biopolymers are widely used in enzyme immobilization. In these supports, the presence of various functional groups, such as -OH, -NH_2, -COOH, -CHO, and epoxy, on the surface allows enzyme immobilization through adsorption and covalent bonding. Additionally, the tendency of these polymers to form gels, along with their different configurations and geometries, makes them highly suitable for entrapment immobilization [50, 51].

Specifically, hydrogels, characterized by their crosslinked polymer network, are renowned for their high water adsorption capacity and the preservation of their three-dimensional structure during swelling, thus effectively entrapping enzymes within [59]. Among the biopolymers frequently utilized in immobilization, chitosan, cellulose, alginate, κ-carrageenan, pectin, and agarose stand out as the most prevalent options (**Figure 4**) [42, 43].

In contrast to natural counterparts, synthetic polymers are artificially synthesized through step-growth or chain-growth polymerization of monomers. Their synthetic origin enables tailored design, modification, and functionalization of monomers to suit specific enzyme immobilization processes and applications. It is noteworthy that monomers dictate the polymer's chemical structure, thereby influencing properties like solubility, porosity, stability, and mechanical attributes. The diverse array of polymers that can be synthesized facilitates the selection of a polymer containing

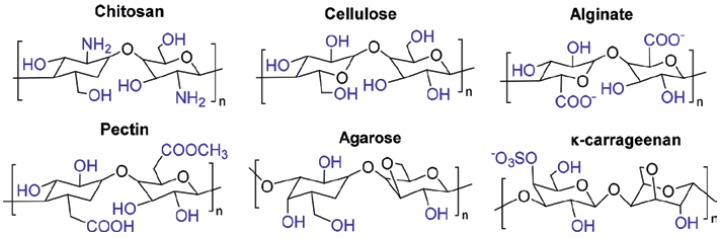


Figure 4.
Structure of the most commonly used biopolymers as polymeric supports.

the most suitable functional groups for anchoring enzymes to the support, aligning with the chosen immobilization strategy. Common functional groups found in synthetic polymers encompass carbonyl (-CO-), carboxylic acid (-COOH), hydroxyl (-OH), epoxy, amine ($-NH_2$), diols, hydrophobic alkyls, and alkylamines ($-NR_2$). Furthermore, surface modification of these supports enhances enzyme affinity while minimizing nonspecific interactions. An additional advantage lies in the ability to regulate spacer length between the polymer matrix and enzymes [60].

Polymeric supports derived from synthetic polymers are extensively employed in immobilization techniques, particularly those involving physical adsorption and covalent bonding. Among the most prevalent are poly(4-vinyl pyridine) (P4VP), polystyrene (PS), polyurethane (PUR), polylactic acid (PLA), polyethylene glycol (PEG), and poly(vinyl alcohol) (PVA) (**Figure 5**) [43, 59].

It's important to note that enzyme immobilization involves establishing weak or covalent interactions between the functional groups on the surface of the polymeric support and those on the amino acids present on the enzyme's surface [49]. Examples of these immobilization methods are depicted in **Table 3**, delineating the functional groups of the polymeric support, the reactive groups on the enzyme, and the nature of the interactions, which are elaborated upon below.

Hydrophobic adsorption immobilization (**Table 3**, entry 1) occurs through interactions between the alkyl groups on the support's surface and the enzyme's hydrophobic regions. Conversely, electrostatic interactions are established between the ionic groups on the support and the enzyme in ionic adsorption immobilization by ion exchange. Within this category, cationic exchange ionic adsorption (**Table 3**, entry 2) involves interactions between protonated amino groups (NH_3^+) on the support and deprotonated carboxylic groups (COO^-) on the side chains of aspartic and glutamic acid residues on the enzyme's surface. Conversely, in anionic exchange adsorption (**Table 3**, entry 3), the anionic groups on the support interact with the cationic groups on the enzyme's lysine and arginine amino acid side chains [46].

Moreover, the existence of electrophilic groups like aldehyde (-CHO) and epoxy groups on the polymeric support's surface facilitates the formation of covalent bonds with nucleophilic groups found on the enzyme's surface (-OH, $-NH_2$, -SH),

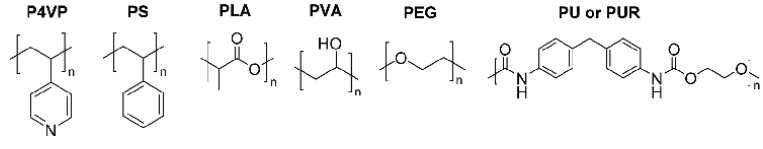


Figure 5.
Structure of the most commonly used synthetic polymers as polymeric supports.

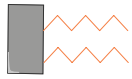
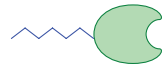
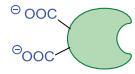
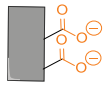
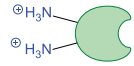
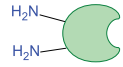
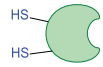
Entry	Functional group of the polymeric support	Surface reactive group of the enzyme	Type of interaction	Immobilization method
1			Hydrophobic interaction	Hydrophobic adsorption
2			Ionic interaction	Ionic adsorption by cation exchange
3			Ionic interaction	Ionic adsorption by anion exchange
4			Covalent bond through epoxy group chemistry	Covalent bond
5			Covalent bonds through aldehyde group chemistry	Covalent bond
6			Disulfide covalent bond	Covalent bond

Table 3.
Some functional groups are present on the surface of the polymeric support and the enzyme, as well as the interaction between these groups.

predominantly engaged in covalent immobilization [58]. In covalent immobilization, the aldehyde groups on the polymeric supports interact with the amino groups ($-NH_2$) on the enzyme's surface, forming secondary amines through Schiff base formation (**Table 3**, entry 5). In contrast, epoxy groups can interact with various nucleophilic groups on the enzyme, primarily hydroxyl (-OH), primary amine ($-NH_2$), and thiol (-SH) groups (**Table 3**, entry 4). When an epoxy group reacts with a primary amine, it forms a secondary amine; with a thiol, it forms a thioether; and with an alcohol, it results in an ether. Heterofunctional supports utilize epoxy groups for immobilization through a two-step process involving ion exchange or hydrophobic adsorption, followed by covalent immobilization based on epoxy group chemistry [58, 61, 62]. The primary amine group ($-NH_2$) is the most significant nucleophilic group found on the enzyme's surface. In fact, proteins typically react through terminal amino groups, specifically the terminal primary amines of the amino acid lysine.

Furthermore, the presence of cysteine in the enzyme provides thiol (-SH) groups capable of forming disulfide bonds with supports modified with -SH groups (**Table 3**, entry 6). Similarly, the presence of tyrosine contributes to an increase in the number of hydroxyl (-OH) groups, which interact with the electrophilic groups on the support [58] (**Table 3**, entry 4).

The modification of polymeric supports is relatively straightforward and allows for the addition of spacer molecules, which act as a bridge between the support's surface and the enzyme's surface, enhancing enzyme mobility. The length of these molecules impacts the enzyme's properties: Longer spacers increase the enzyme's conformational flexibility, thereby enhancing mobility, while shorter ones confer thermal stability and reduce the likelihood of enzyme detachment from the support [58].

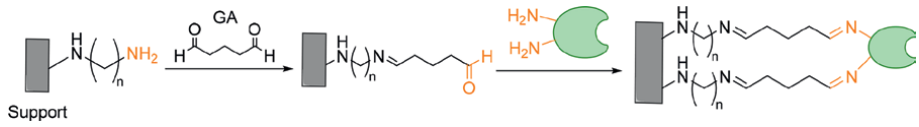


Figure 6.
Activation of the amino groups of the polymeric support with GA and covalent immobilization of the enzyme.

On the other hand, polymeric supports may feature nucleophilic groups on their surface, predominantly amino (-NH_2) and hydroxyl (-OH) groups, facilitating support functionalization with electrophilic groups for enzyme anchoring via covalent bonds. Supports with amino groups are activated using difunctional reagents like glutaraldehyde (GA), enabling the incorporation of aldehyde groups on the surface capable of reacting with amino groups on the enzyme, forming Schiff bases (depicted in **Figure 6**). The resulting imine can then be stabilized by reduction with $NaBH_4$, minimizing enzyme leakage but potentially compromising enzyme activity. Conversely, supports with hydroxyl groups can be activated with cyanogen bromide (BrCN), which reacts with these groups to form imidocarbonates, subsequently engaging with amino groups on enzymes (particularly lysine) to forge covalent bonds [61].

4.1 Immobilization of β-galactosidase on polymeric supports

As previously mentioned, β-galactosidase functions as a hydrolase, an enzyme that catalyzes the breakdown of chemical bonds by adding a water molecule. In the case of lactose, β-galactosidase breaks the β-(1 → 4)-glucosidic bond present in this disaccharide. Notably, this enzyme also exhibits transglycosidic activity, enabling the transfer of galactose from a galactosyl donor compound to an acceptor compound, leading to the formation of oligosaccharides or glucosides [63]. Industrially, β-galactosidase holds significant importance, particularly in the production of lactose-free products within the food industry and the synthesis of galacto-oligosaccharides (GOS). These GOS serve as non-digestible prebiotics, fostering the growth of beneficial bacteria in the intestine and contributing to human health by modulating immune system activity and potentially preventing conditions like cancer, among other positive effects [63]. Given its pivotal role in industrial processes, considerable research and development efforts have been dedicated to creating polymeric supports for its immobilization.

The structural features, composition, and arrangement of amino acids on the surface of β-galactosidase facilitate its immobilization through various methods such as adsorption, entrapment, covalent bonding, and crosslinking. Notably, the presence of specific amino acid residues like aspartic acid, glutamic acid, lysine, and arginine on the enzyme's surface plays a crucial role in its immobilization onto ion-exchange resins through ionic adsorption. For instance, the carboxyl (-COOH) groups located on the side chains of aspartic and glutamic acids can undergo deprotonation by adjusting the pH, allowing them to electrostatically interact with cationic groups present on the surface of the polymeric support. Conversely, the amino groups (-NH_2) found on the side chains of lysine and arginine can be protonated and interact with anionic groups on the support. On the other hand, covalent bonding to the support is achieved through various functional groups, including the amino groups on lysine side chains, the terminal amino groups of the enzyme's subunits, the hydroxyl groups on tyrosine residues, the thiol groups of cysteine, and the imidazole groups of histidine (**Figure 7**) [58].

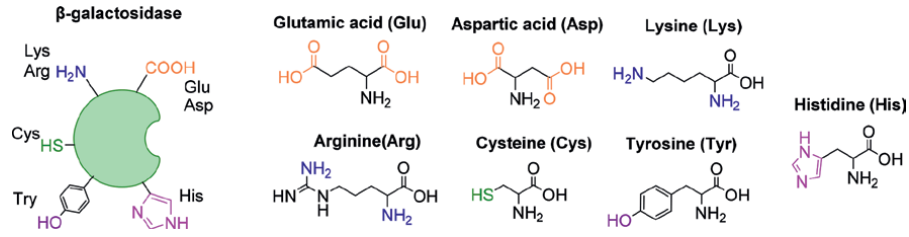


Figure 7.
Schematic representation of β-galactosidase and the amino acids involved in its immobilization.

4.1.1 Immobilization of β-galactosidase by adsorption

The even distribution of anionic amino acids, such as glutamic acid and aspartic acid, across the surface of β-galactosidase facilitates its immobilization through ionic adsorption onto ion-exchange resins [64, 65]. This method was successfully applied in the immobilization of β-galactosidase from *A. oryzae* on an anion-exchange resin functionalized with quaternary ammonium groups (**Figure 8A**) [65]. The process resulted in a 76% yield in enzyme immobilization under conditions of 30°C temperature and a pH of 7. The objective of this immobilization was to synthesize lactulose, a bioactive disaccharide widely utilized in pharmaceuticals and as a prebiotic ingredient in food products (**Figure 8B**).

In another study, β-galactosidase from *A. oryzae* was immobilized on a commercial anion-exchange resin, Duolite™ A568 (phenol-formaldehyde resin) [66]. This method involved activating tertiary amine groups present on the support's surface in an acidic medium. Optimization was achieved by combining ionic adsorption with enzyme crosslinking using GA. Notably, the adsorption-based immobilization retained 51% of the initial enzymatic activity after 30 operational cycles, while the adsorption-crosslinking immobilization retained 90%.

Anion-exchange resins stand out as the preferred polymeric support for the ionic adsorption immobilization of β-galactosidase due to their ability to yield high rates of

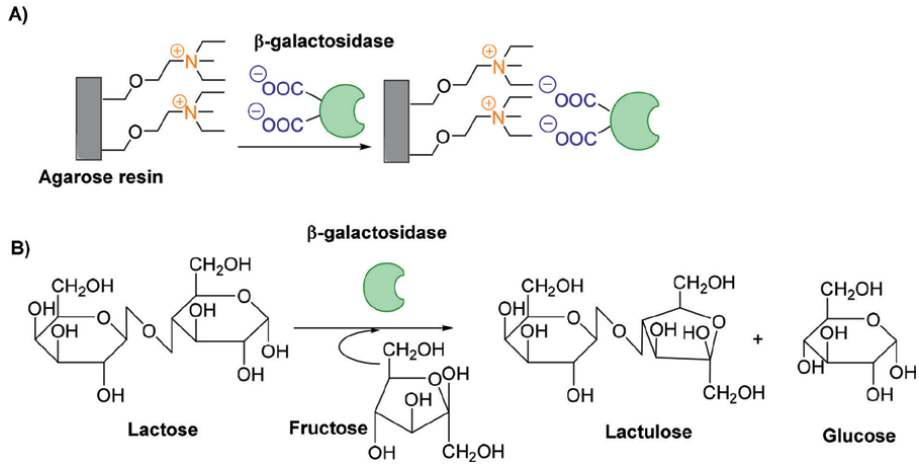


Figure 8.
Ionic adsorption immobilization of β-galactosidase: (A) immobilization system and (B) lactulose synthesis reaction carried out by the enzyme-support system.

enzyme immobilization. Nevertheless, variations in the pH of the medium can trigger enzyme desorption from the support, underscoring the criticality of pH control during both the immobilization process and lactose hydrolysis [67].

4.1.2 Immobilization of β-galactosidase by entrapment

The entrapment immobilization of β-galactosidase commonly utilizes hydrogels based on natural polymers, prized for their biocompatibility, biodegradability, and lack of toxicity. For instance, a crosslinked chitosan hydrogel incorporating acrylic acid and *N′*,*N′*-methylenebisacrylamide has been employed to hydrolyze lactose in both commercial lactose solution and UHT milk [68]. Over 10 lactose hydrolysis operational cycles in the commercial lactose solution, the activity of the immobilized enzyme decreased from 98% to 56%, while in UHT milk, it decreased from 98% to 72%. Similarly, Arabic gum hydrogels crosslinked with acrylamide have shown promise in lactose hydrolysis since, after three operational cycles, enzymatic activity was recorded at 53% and 94% in standard lactose and UHT milk, respectively [68, 69].

It's also commonplace to utilize hydrogels formed by the combination of two biopolymers. For instance, β-galactosidase has been immobilized in a pectin hydrogel incorporating varying percentages of pine residue (5% and 10%) [70]. Pine residue, comprising cellulose, hemicellulose, and lignin, bolsters the mechanical resilience of polysaccharide-based hydrogels owing to its chemical composition. Furthermore, its affordability, biocompatibility, and eco-friendly attributes make it particularly intriguing to develop solid supports. Immobilization studies with this composite revealed that at pH 4.0 and after 600 minutes, the enzyme's immobilization capacity was 181 mg of enzyme per gram of dry hydrogel for the 5% pine residue and 183 mg enzyme per gram of dry hydrogel for the 10% pine residue. However, at pH 5.6, the immobilization capacity increased to 220 and 219 mg of enzyme per gram of dry hydrogel for the 5% and 10% pine residue, respectively. An enzyme immobilization within a pectin hydrogel was also conducted to contrast the outcomes with those obtained from the prior support. The immobilization capacity within this pectin hydrogel was observed to be 242 and 183 mg of enzyme per gram of dry hydrogel at pH 4.0 and 5.6, respectively. It's essential to consider that the carboxyl groups of the pectin hydrogel deprotonate at pH values surpassing the pK_a of pectin, which stands at 3.5. Conversely, the cationic groups on the surface of β-galactosidase prevail at pH values below its isoelectric point, which is 4.8. Consequently, the higher immobilization capacity of the pectin hydrogel at pH 4.0 compared to pH 5.6 is attributed to the robust electrostatic attractions established between the deprotonated carboxyl groups of the hydrogel and the protonated groups of the enzyme, thereby enhancing the stability of the enzyme-support bond. In contrast, at pH 5.6, intense electrostatic repulsion ensues between the negatively charged groups of the hydrogel and the enzyme, resulting in destabilized immobilization. However, a contrasting pattern emerges with the pectin/pine residue hydrogel, as immobilization demonstrates greater stability at pH 5.6 compared to pH 4.0. This shift arises from the pine residue, which mitigates repulsion between the negatively charged enzyme groups and the pectin by positioning itself within the hydrogel pores, thus increasing the separation between these groups. Similarly, in another study [71], lactose hydrolysis was conducted utilizing β-galactosidase immobilized within a hydrogel composed of alginate and gelatin crosslinked with genipin, a natural crosslinking agent derived from gardenia fruit. The immobilized enzyme exhibited remarkable longevity, retaining 90% of its initial activity after 11 operational cycles in a batch reactor and maintaining 80% of its initial activity even after 175 days of storage in a freezer.

Mostly, hydrogels made from natural polymers are chosen for the immobilization of β-galactosidase, although hydrogels made from synthetic polymers can also be employed. One such example is the use of LentiKats®, a PVA hydrogel, for the immobilization of β-galactosidase from *K. lactis* and *A. oryzae* [72]. The study delved into the potential of this enzyme-support system in lactose hydrolysis to yield the monosaccharide D-galactose. Notably, the immobilized enzymes were employed in tandem with yeasts: Initially, β-galactosidase catalyzed lactose hydrolysis, followed by yeast-mediated fermentation of glucose to yield galactose. Results revealed that β-galactosidase from *A. oryzae* retained 80% of its initial activity after 466 operational hours in a batch reactor, generating D-galactose at a rate of 1.9 g/L h. Conversely, under identical conditions, β-galactosidase from *K. lactis* exhibited a lower retention of initial activity at 50%, yet yielded a higher D-galactose production rate of 3 g/L h. Another noteworthy example involves the immobilization of β-galactosidase from *A. oryzae* within cryogels, which in this case, are gels formed at sub-zero temperatures, composed of polyacrylamide (PAM) and poly(2-hydroxyethyl methacrylate) (HEMA) chelated with Fe^{3+} [73]. This study evaluated the enzyme-support system's efficacy in lactose hydrolysis, revealing that the immobilized enzyme exhibited optimal conditions at pH 3.0 and preferred temperatures between 60°C and 65°C. Impressively, it retained 65% of its initial activity after 25 operational cycles and experienced a modest decline of 29% in activity following 70 days of storage.

These findings underscore the prominence of supports based on polysaccharide hydrogels in β-galactosidase immobilization for diverse applications. Their appeal lies in their biocompatibility, substantial immobilization capacity, facilitation of environmentally friendly support utilization, straightforward preparation, and ability to maintain enzymatic activity across numerous operational cycles.

4.1.3 Immobilization of β-galactosidase by covalent bonding

As discussed earlier, covalent immobilization of β-galactosidase typically involves the interaction between nucleophilic groups on the enzyme and electrophilic groups on the polymeric support. Polymeric supports featuring epoxy groups on their surface react with amine, hydroxyl, and thiol groups on the enzyme's surface, forming covalent bonds of secondary amine, ether, and thioether types. For instance, for lactose hydrolysis in milk, a recent study describes the immobilization of *Escherichia coli* β-galactosidase using an acrylic UV-cured polymeric film functionalized with epoxy groups [74]. The enzyme was anchored to the polymeric film through the reaction of the epoxy groups on the support with the amine groups present on the enzyme's surface. The immobilized β-galactosidase exhibited an optimal pH of 6.5 and temperature of 60°C, retaining 51% of its initial activity after 12 operational cycles of lactose hydrolysis. Regarding the support composition, the polymeric film comprises glycidyl methacrylate monomers (20% by weight), trimethylolpropane triacrylate (15% by weight), and poly(ethylene glycol) methyl ether acrylate polymer (65% by weight) (**Figure 9A**). This support is characterized by its excellent resistance to chemicals and abrasion, as well as thermal insulation properties, high thermal and mechanical stability, and strong adhesion to substrates of diverse natures.

Another example of β-galactosidase immobilized in a film-shaped polymeric support was described in the work by Vallejo-García et al. [75]. In this study, β-galactosidase from *A. oryzae* was covalently immobilized via azo linkages in highly manageable polyacrylic films and subsequently employed to hydrolyze lactose from commercial milk (**Figure 9B**). Results demonstrated that these films were capable of

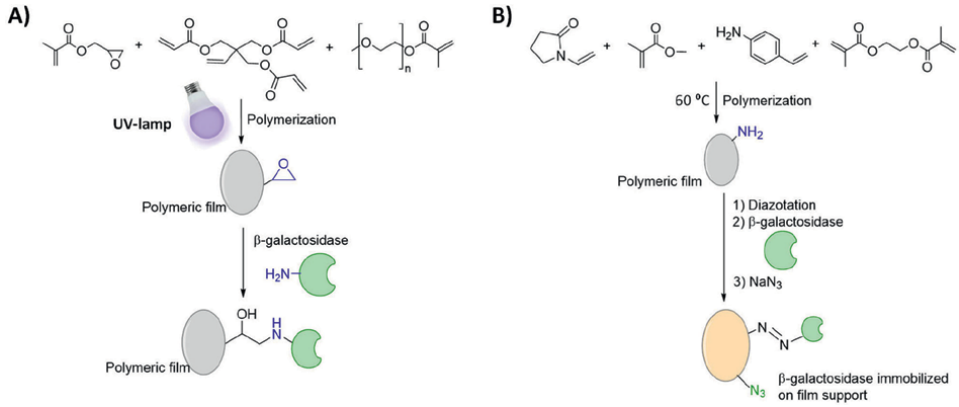


Figure 9.
Immobilization of β-galactosidase on a polymeric film with epoxy groups.

producing lactose-free milk (with concentrations below 0.1 g/100 mL) after 20 hours of incubation at 25°C or 6 hours at 55°C. However, at 4°C, the final lactose concentration after 30 hours was not sufficiently low to be considered lactose-free but rather low-lactose milk. Additionally, the material's operational and storage stability is noteworthy, as it retained its ability to hydrolyze lactose in milk even after 10 cycles and 1 month of storage without any loss of activity, which was attributed to the covalent immobilization method.

When considering commercially available supports, an illustration can be found in the immobilization of β-galactosidase from *A. oryzae* on a commercial polymeric support named epoxy Immobead 150® (Immobead-Gal, **Figure 10A**) [76]. Additionally, the enzyme was immobilized on the same support after modifying it with aldehyde groups, achieved through the reaction of the epoxy groups on the surface with GA, resulting in Immobead-Glu-Gal (**Figure 10B**). The immobilization process on the unmodified support entailed the formation of covalent bonds between the epoxy groups on the support and the amino groups of β-galactosidase (**Figure 10A**). Conversely, on the modified Immobead-Glu support, covalent bonds were established between the aldehyde groups on the support and the amino groups of the enzyme (**Figure 10B**).

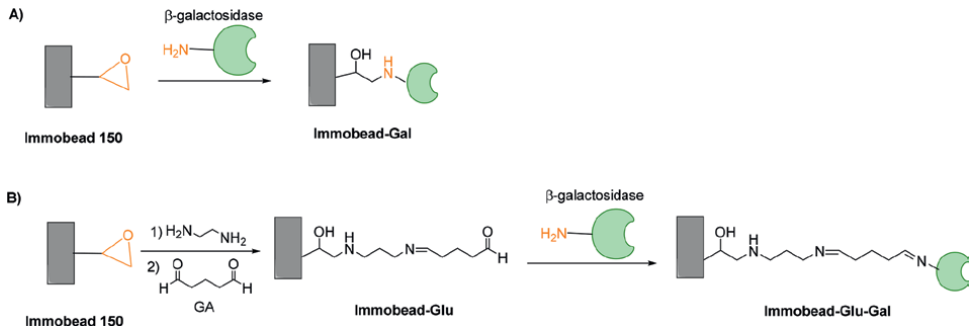


Figure 10.
Immobilization of β-galactosidase by covalent bonding: (A) Immobead 150® support and (B) Immobead-Glu support.

The effectiveness of immobilization was assessed through lactose hydrolysis in various substrates, including an artificial lactose solution, whey filtrate, cheese whey, and skim milk. Both supports exhibited a yield of over 75% in enzyme immobilization, with the enzyme retaining 50% of its initial activity after 20 operational cycles using the lactose solution. Notably, the optimal pH shifted from 5.0 for the free enzyme to 6.0 for the immobilized enzyme. Furthermore, the immobilized enzyme displayed significantly higher activity at 70°C compared to its free counterpart.

In addition to these supports, there is a growing interest in enzymatic immobilization on hybrid supports formed by combining polymeric materials with nanomaterials, leveraging both materials' superior chemical, physical, and mechanical properties. These supports have been used for the covalent immobilization of β-galactosidase in applications requiring lactose hydrolysis. One notable example is the hybrid support Fe_3O_4@PDA@DAPEG-GA, developed for the immobilization of β-galactosidase from *A. oryzae* (**Figure 11**) [77]. Comprising Fe_3O_4 nanoparticles along with the polymers polydopamine (PDA) and polyethylene glycol diamine (DAPEG), this support's surface is functionalized with GA. GA reacts with the amino groups of lysine and arginine residues on the enzyme's surface to achieve immobilization. The enzyme immobilized on this support displayed an optimum pH of 6.0 and an optimum temperature of 45°C, which is 15°C higher than that of the free enzyme. Moreover, the enzyme retained 94% of its initial activity in a lactose hydrolysis process after 96 hours in a continuous reactor.

Up to this point, all the supports mentioned for the covalent immobilization of β-galactosidase are composed of synthetic polymers, but biopolymers can also be used. β-Galactosidase from *A. oryzae* was immobilized on chitosan support, where the support was either fully or partially functionalized with aldehyde groups [78]. In the fully modified support, aldehyde groups are introduced by reacting the surface groups of the support with GA or epichlorohydrin (ECH). The enzyme is then immobilized through the formation of covalent bonds between the amino groups on the enzyme's surface and the aldehyde groups on the support's surface at pH 10, to deprotonate ε-amino groups of the lysine (**Figure 12A**). In contrast, the partially functionalized surface support distinguishes aldehyde groups introduced by reaction with ECH and amine groups previously activated in an acidic medium (pH 5.5) (**Figure 12B**). In this support, immobilization occurs in two stages. Initially, at pH 5.5, the enzyme is immobilized by ionic adsorption between the protonated amino groups on the support's surface and the carboxylate groups on the enzyme's surface.

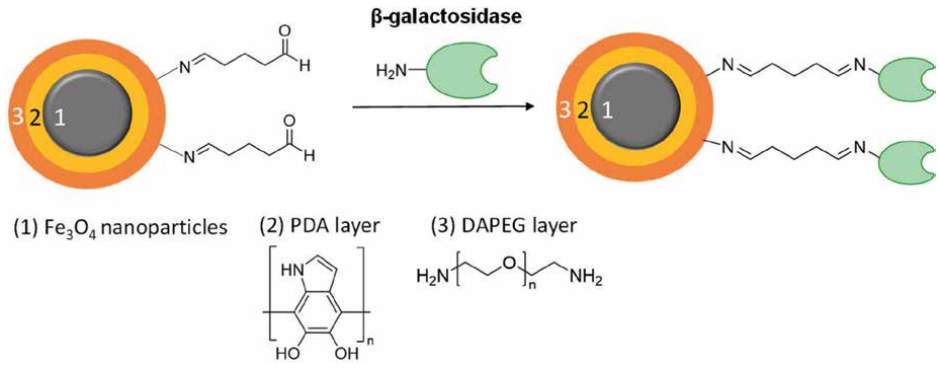


Figure 11.
β-Galactosidase immobilization on the Fe_3O_4@PDA@DAPEG-GA hybrid support.

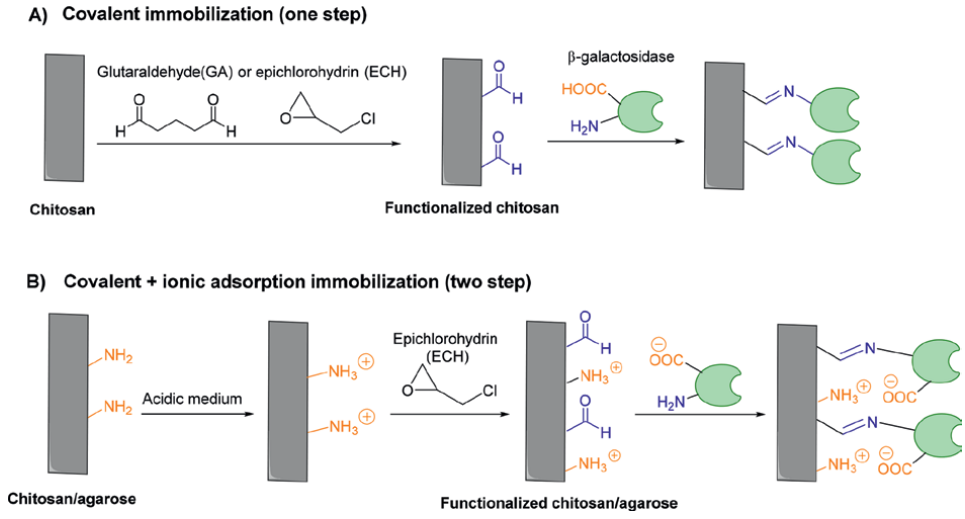


Figure 12.
Immobilization of β-galactosidase on biopolymers: (A) support fully modified with aldehyde groups and (B) support partially modified with aldehyde groups.

Subsequently, at pH 10, covalent immobilization occurs through the formation of covalent bonds between the aldehyde groups on the support's surface and the amino groups on the surface of β-galactosidase (**Figure 12B**).

The percentages of β-galactosidase immobilization on supports fully functionalized with GA or ECH, and on the partially ECH-modified support were 29%, 6%, and 42%, respectively. When the support is fully functionalized with GA, a higher immobilization efficiency is achieved compared to when ECH is used. This is attributed to the versatile chemistry of GA and its various degrees of polymerization, enabling multiple mechanisms for enzyme immobilization, besides Schiff base formation. However, a higher yield is obtained in the support partially functionalized with ECH compared to the previous supports. Here, preliminary immobilization by ionic adsorption occurs, facilitating interaction between the amino groups on the enzyme's surface and the aldehyde groups on the support's surface, leading to intramolecular covalent bond formation and a higher immobilization capacity. Additionally, the partially functionalized support exhibited greater thermal stability, likely due to multipoint attachment with the support and/or a different enzyme orientation.

Given the robust stability of β-galactosidase during operational conditions, covalent immobilization onto polymeric supports is extensively employed in lactose-free product production, ensuring sustained enzymatic activity across numerous operational cycles. Nevertheless, the fabrication processes of these supports can sometimes be intricate. Furthermore, a full comprehension of the covalent bond formation mechanism between the support surface and the enzyme surface is imperative to forestall any alterations to the enzyme's active center, which could lead to a decline in catalytic activity.

5. Conclusions and future perspectives

There is a growing interest in producing lactose-free products via enzymatic hydrolysis using β-galactosidase immobilization methods on supports. This approach

allows lactose-intolerant individuals to incorporate these essential products into their diets without compromising their health. Enzymatic immobilization stands out among other lactose separation or hydrolysis methods due to its ability to reuse the enzyme across multiple operational cycles, resulting in cost reduction in industrial production, ensuring enzyme stability under operational conditions, and preserving the enzyme's conformation and catalytic activity. Additionally, the wide range of available supports offers the flexibility to select the most suitable support for the immobilization process, the enzyme, and the specific application.

In this context, it's crucial to emphasize the pivotal role of polymeric supports, both natural and synthetic, in enzymatic immobilization. Their utilization presents numerous advantages, including biocompatibility, contribution to the development of environmentally friendly supports, versatility, availability of various functional groups for enzyme anchoring through different immobilization methods, and exceptional chemical, mechanical, and biological properties.

Industrially, transitioning from batch processing to continuous processing holds potential economic and environmental advantages, primarily due to the recovery of enzymes, which would reduce the overall cost of enzymatic hydrolysis. However, the commercial development of continuous enzymatic hydrolysis faces obstacles related to scaling up batch reactors and the recovery and cleaning of immobilized enzymes. Additionally, the recent surge in patents related to enzyme immobilization, particularly β-galactosidase, presents a promising advancement. Further exploration into the feasibility and development of large-scale bioreactors utilizing immobilized β-galactosidase that can be efficiently reused is imperative. Thus, there is a crucial need for research focused on theoretically scaling up enzymatic bioreactors to advance the commercialization of this process. Continuous efforts in developing β-galactosidase immobilization processes on polymeric supports are essential for designing efficient and economically viable industrial processes for lactose-free product production.

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Conflict of interest

The authors declare no conflict of interest.

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Author details

Jorge Lucas Vallejo-García, Alessandra Cutillo-Foraster, Ana Arnaiz, Saúl Vallejos, José M. García, María Asunción Muñoz Santamaría and Miriam Trigo-López*
Polymer Research Group, Facultad de Ciencias, Departamento de Química, Universidad de Burgos, Burgos, Spain

*Address all correspondence to: mtrigo@ubu.es

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Chapter 4

Interactions between Lactose-Proteins-Minerals in Dairy Systems: A Review

Yuanyuan Zhao, Juhi Saxena, Tuyen Truong and Jayani Chandrapala

Abstract

Milk and dairy products are complex matrices rich in diverse macronutrients and micronutrients. Lactose, a key component, interacts with milk proteins primarily through hydrogen bonding, while proteins interact via hydrogen bonds, hydrophobic interactions, and electrostatic forces. These interactions, along with mineral-protein interactions, significantly influence the functionality and stability of dairy products. The physical state of lactose and the nature of mineral interactions—shaped by the type, concentration, and processing conditions—can trigger reactions that alter the physicochemical properties of the system. Additionally, the stability of these systems is affected by the specific types and concentrations of proteins and minerals involved. Processing steps such as thermal treatment, concentration, fermentation, and drying, as well as non-thermal technologies like high-intensity ultrasound, further modify these interactions, impacting product quality and storage stability. Understanding these intricate relationships is crucial for optimizing the design and formulation of dairy products. This review examines the mechanisms of lactose-protein, lactose-mineral, and protein-mineral interactions in both liquid and solid systems, highlighting the significant implications these interactions have on processing and product stability.

Keywords: dairy, lactose, interactions, caseins, whey proteins, minerals

1. Introduction

Milk and other dairy products are essential components of human diet as they are rich sources of high-quality proteins, lactose, vitamins, and minerals [1, 2]. However, technological and product quality challenges are often encountered with both liquid and solid-state dairy systems. These challenges can arise from the complex interactions between milk components.

Lactose, the primary sugar source in milk, is a commonly used ingredient or additive in the food and dairy industry due to its unique physiochemical properties [3–6]. However, the behavior of lactose in dairy products can significantly affect the storage stability and quality of certain powder products, such as milk protein powders

and infant formula [3, 5–8]. For instance, the degree of lactose crystallization causes stickiness, caking, collapse, and accelerations of the Maillard reaction in lactose-containing milk powders during storage [7] as amorphous lactose is metastable, thermodynamically unstable, and hygroscopic [9].

The mineral content of milk, although constituting a relatively small fraction at approximately 8–9 g/L, includes essential cations like calcium, magnesium, sodium, and potassium, as well as critical anions including inorganic phosphate, citrate, and chloride. Of these, calcium is gaining particular attention for its enrichment in food and dairy products, aligning with the increasing recognition of high calcium intake [10–12]. However, fortifying milk with calcium can be challenging, especially if the product needs to be heat-treated [10]. The added calcium ions can cause significant protein instability by decreasing the net charge of the proteins, resulting in protein aggregation during processing and storage [13, 14]. This instability can ultimately compromise the quality and shelf life of calcium-fortified milk products.

In addition to being an excellent source of nutrition, milk proteins and their interactions with other components play an important role in conferring desirable functional properties to final dairy products [15, 16]. It is widely known that the processing of milk proteins through heat or high pressure can result in a modification to protein structure, resulting in altered interactions between the proteins and other milk components [17–19]. However, these interactions are influenced by various factors, including pH, temperature, moisture content, milk protein composition, pressure, drying procedures, processing parameters, and the presence of other ingredients [4]. For example, low pH can cause caseins to lose their steric stability and induce coagulation, while high temperature can cause whey proteins to denature and facilitate aggregation. The extent of these changes in functionality is a function of the presence of lactose and minerals [15, 20, 21]. Another instance, dairy products are highly susceptible to Maillard reaction based on their composition (lactose and lysine in caseins and whey proteins) and under low relative humidity conditions [22–25]. On the other hand, studies have shown that the presence of lactose has a protective effect on the thermal denaturation of whey proteins [26]. A typical example of a solid-state interaction between lactose and protein is that the amorphous phase of lactose stabilizes the structure of proteins during spray drying and freeze drying [7]. Lactose maintains or increases the hydration of protein molecules, therefore contributing to their stability. Meanwhile, the presence of milk proteins can inhibit or delay lactose crystallization in milk powders due to the development of interactions in the solid state between proteins and amorphous sugars [27, 28]. Besides, minerals as impurities in lactose solutions would influence lactose crystallization in a direction that was depended on the type and the concentration of the salt present [29]. For example, the presence of calcium can directly affect the behavior of lactose, hindering the removal of water surrounding lactose molecules and inhibiting the overall crystallization process [30, 31].

Furthermore, emerging dairy processing technologies such as ultrasound have great influence on these component interactions. For instance, physical shear forces generated through acoustic cavitation with the application of high intensity ultrasound can disrupt heat-induced protein aggregates by breaking intermolecular and intramolecular protein bonds. This disruption prevents the formation of aggregates during subsequent heating, reducing heat-induced viscosity increase, and enhancing heat stability [32]. However, these effects depend on the system's composition, pH, temperature, sonication time, amplitude, and the presence or absence of applied pulses during treatment.

Although the factors controlling the physical attributes of dairy products are well documented with various processing techniques, further exploration of the interactions between milk proteins, lactose, and minerals that occur during the dairy manufacturing processes is needed. The current body of knowledge on structure-function relationships and interactions in lactose-protein-mineral systems is limited, particularly in understanding the precise molecular mechanisms and the impact of various processing methods. Thus, it is important to expand on this topic to better understand how these interactions affect the functionality, stability, and nutritional quality of the final dairy products. This review covers the fundamental properties of individual dairy components and their behavior in milk systems. Specifically, it focuses on the interactions between milk proteins, lactose, and minerals, particularly calcium, along with the major implications of these interactions in both liquid and solid-state dairy systems. Liquid-state protein-protein interactions through covalent, hydrophobic, and electrostatic interactions, as well as lactose-protein interactions through hydrophobic interactions and milk protein, lactose, and mineral complexations, will be discussed in detail. Furthermore, lactose crystallization and minor reactions associated with lactose, as influenced by the presence of minerals and proteins in the solid state, will be examined. By studying these interactions in detail, a comprehensive understanding of the factors that affect the physical and functional properties of dairy products can be gained. This knowledge can be used to develop more effective manufacturing processes and improve the overall quality of dairy products.

1.1 Milk components and their applications

1.1.1 Lactose

Lactose, a disaccharide made up of glucose and galactose molecules, is the primary carbohydrate found in milk. Lactose ($C_{12}H_{22}O_{11}$) is naturally present in milk of most mammals, albeit in varying concentrations depending on the species. For instance, human milk contains approximately 7% lactose, while cow and goat milks contain around 4.5–5% lactose [2, 5]. In milk, lactose exists in two basic configurations, α- and β-lactose, which are continuously converted into each other through mutarotation [2]. These two forms differ from the position of the hydroxyl group on the carbon atom of the glucose molecule. There are marked differences between α- and β-lactose, especially in solubility and crystallization behavior. For instance, α-lactose is much less soluble and crystallizes as a monohydrate, while β-lactose is more soluble and forms anhydrous crystals [33].

Physicochemical behavior of lactose is found to have a significant impact on various dairy products and the properties of ingredients during processing and storage [3, 4, 29]. Lactose is less soluble than other sugars, such as sucrose and glucose [3, 34]. In general, lactose solubility is lower in dairy systems compared to pure lactose solutions. This is because the presence of components with high water binding capacity in dairy systems (e.g., proteins and polysaccharides) increases the competition for water, thereby reducing the solubility of lactose [35]. Moreover, the presence of salts influences the solubility of lactose by changing the structure of surrounding water molecules. For instance, adding sodium phosphate can reduce lactose solubility [36], while the addition of potassium phosphate can increase the solubility of lactose [37].

Like other sugars, lactose molecules nucleate and crystallize when the concentration of this sugar overcomes its maximum solubility at a specific temperature. The dairy industry applies this principle to crystallize lactose from whey, a by-product of

cheese-making and yoghurt production [30]. Lactose crystallization consists of a set of complex reactions that strongly depend on the experimental conditions used (temperature, agitation, and supersaturation) and, most importantly, the composition of the samples (water, minerals, soluble proteins) [5]. Various lactose crystal forms can theoretically be formed; but α-lactose monohydrate is the main crystalline form produced from dairy products under normal industrial conditions [5]. These α-lactose monohydrate crystals are very hard, slighly hygroscopic, and dissolve slowly, rendering it as the most stable form [3]. However, during spray drying, the rapid removal of water causes lactose to dry under its saturation point, thus leading to a rapid increase in its viscosity. Hence, amorphous lactose in a glassy state is formed, which also contains some moisture. For instance, lactose in infant formula (IF) powders is present in amorphous or crystalline forms, with the former being predominant [38]. Amorphous lactose is hygroscopic in nature, which makes it prone to plasticization by water [39]. The instability of amorphous lactose in dairy powders is strongly related to many storage and handling problems of these powders. As a result, control of processing and storage conditions are crucial to control the lactose behavior in ingredients.

1.1.2 Milk proteins

Milk proteins comprise two main groups: caseins and whey proteins. They are widely used in the food industry as functional ingredients due to their high nutritional and functional properties, such as water binding, foaming, emulsification, and gelation [40].

1.1.2.1 Casein

Casein constitutes approximately 80% of the total protein in bovine milk. They are present as macromolecular aggregates of proteins and minerals forming colloidal particles named micelles with a mean size of 150 nm [18, 21, 41]. A micelle contains about 94% protein and 6% colloidal calcium phosphate (CCP, which include calcium, magnesium, phosphate, and citrate). They are proline-rich, open-structured rheomorphic proteins, which have distinct hydrophobic and hydrophilic domains [42, 43]. **Table 1** summarizes key physicochemical features of caseins against whey proteins, highlighting their unique structure and properties. Casein micelles have a unique structure that allows them to interact with other milk components, such as whey proteins, lactose, and minerals, and contribute to the texture and stability of dairy products [40]. Casein molecules can hardly be denatured because they have little secondary and tertiary structure [35]. There are four individual types of casein molecules, the α_{s1}-, α_{s2}-, β-, and κ-CN in the approximate ratio of 4:1:3.5:1.5, respectively. Casein proteins can be divided into two groups such as the calcium-sensitive and the non-calcium-sensitive. α_{s1}-, α_{s2}-, and β-CN are classified as calcium-sensitive caseins, whereas κ-CN is highly phosphorylated and thus calcium-sensitive [41, 45].

1.1.2.2 Whey proteins

Whey proteins have been widely used as an ingredient for many food and dietary supplement products, such as nutritional bars, infant formula, and protein and/or energy drinks (**Table 2**). Whey proteins are globular proteins with high levels of α-helix structure. The acidic-basic and hydrophobic-hydrophilic amino acids are distributed in a balanced form [46]. Whey proteins are characterized with a well-defined

Properties	Whey proteins	Caseins
Structure	Well-defined tertiary and quaternary structure	Lack well-defined secondary, tertiary, and quaternary structure; possess random coiled structure
Amino acid composition	Relatively high in sulfur-containing amino acids; low in proline	Low in sulfur-containing amino acids; high in proline
Physical state	Exist as globular proteins in the form of monomer-octamers, depending on pH	Exist as large colloidal aggregates called casein micelles
Solubility at pH 4.6	Soluble at pH 4.6	Insoluble at pH 4.6
Heat stability	Heat-labile (can be completely denatured, particularly when heating at 90°C or high)	Very heat-stable (can withstand severe heat treatment such as sterilization, ultrahigh temperature, or retort processing)

Table 1.
Comparison of selected physiochemical properties of casein and whey proteins based on information from [44].

Functionality	Description	Main mechanism	Applications
Solubility	Ability to dissolve in a solvent	Hydrophilicity	Recombined, UHT and sterilized milk, soups and sauces, infant and clinical nutrition, sports beverages, protein-fortified beverages
Water holding capacity	Interaction with product components to provide high water-holding capacity	Hydrogen bonding	Meat products, bakery products, confectionary, frozen desserts, prepared foods
Fat holding capacity	Flavor interactions/ flavor binding and fat retention	Hydrophobic bonding	Bakery products, yoghurt
Gelation	Gel formation	Protein-protein network formation	Meat, sausages, pasta, bakery, yoghurt
Foaming	Protein adsorption at the interface and coating of air cells	Interfacial adsorption, film formation	Whipped cream and toppings, cakes, mousse, meringues
Emulsification	Protein adsorption at the interface and coating of oil droplets	Interfacial adsorption, film formation	Salad dressings, soups, sauces, and dips, sausage (meat emulsions), ice cream, processed cheese, coffee whitener

Table 2.
Functional properties and underlying mechanisms for the applications of proteins in food products. Reprinted with permission from [19].

tertiary structure, and they are heat labile. During food processing and storage, whey proteins are liable to denaturation followed by aggregation. The aggregation of proteins in a food matrix can result in dramatic changes in the global structure and texture.

Whey proteins include β-lactoglobulin (β-Lg), α-lactalbumin (α-Lac), immunoglobulins (IG), bovine serum albumin (BSA), bovine lactoferrin (BLF), and lactoperoxidase (LP), together with other minor components [47, 48]. β-Lg is the major whey protein in bovine milk, representing about 50% of total whey protein or 12% of

the total protein of milk. When in isolated form, it exhibits a low solubility and a low ionic strength. α-Lac represents about 20–25% of the proteins of bovine whey and is a calcium-binding protein [40, 49, 50].

1.1.3 Minerals

Milk minerals are crucial for human health and development, and they play a critical role in dairy processing, particularly in cheese-making and other processes that involve salt-protein interactions [51]. In milk, these ions are associated between themselves and with proteins. For instance, Schuck et al. [52] reported that adding mineral salts to milk changed the casein micelle structure and micellar mineral composition, causing significant variations in water transfer during the drying process. Thus, the mineral content has a pronounced effect on the technological properties of milk, as it affects its susceptibility to renneting, fouling of heat exchanges, gelation, and sedimentation [53]. Minerals are present in two primary forms in milk, soluble (or diffusible), and micellar (**Table 3**). Soluble minerals are present in a highly dynamic equilibrium between ionic and associate forms, mainly represented by citrate, phosphate, sulfate, and chloride salts [55]. The mineral balance, mainly the divalent cations of Ca and Mg and phosphate, is affected by changes in composition and milk processing parameters such as pH and temperature [55].

1.1.3.1 Calcium

Calcium (Ca) is an essential mineral abundant in milk and dairy products. It plays a crucial role in various physiological functions in the body, including the

Minerals	**Total concentration (mM)**	**Concentration (mM)**	
Calcium	29.4–30	Micellar	19–20.2
		Soluble	9–10
		Ionic	1.9–3.0
Magnesium	3.42–5.43	Micellar	1.23–1.95
		Soluble	2.18–3.47
		Ionic	0.54–0.86
Inorganic phosphate	21	Micellar	9.7
		Micellar Ca:P	2.08
		Soluble	11.2
		Ionic	0.82–0.85
Citrate	9.32	Micellar	0.9–1
		Soluble	8.2
Soluble sodium	24.2		
Soluble potassium	34.7		
Soluble chloride	30.2		

Table 3.
Minerals distribution in bovine milk. Reprinted with permission from [54].

development and maintenance of strong bones and teeth, as well as nerve and muscle function [56, 57]. In dairy production, Ca is often used as a coagulant in cheese-making. It helps to give the cheese its firm texture and plays a vital role in the development of the cheese curd. Ca can also be added to dairy products as a nutrient supplement to improve their nutritional profile [53, 58].

On average, the total Ca content in bovine milk is 30 mM, of which approximately 66% is bound to the casein micelles, while 33% is in the serum phase [56, 59, 60]. Ca in the serum phase is largely soluble and associated with serum phosphate and citrate, and only a small portion is ionic (~ 10% of total Ca). Micellar Ca can locate both on the surface and inside the casein micelles. Ca located on the surface helps micelle aggregation during the formation of the Para casein reticulum during cheese-making [61]. At the same time, Ca is present in the internal part of casein micelles as Ca phosphate, also defined as colloidal Ca (CCP; $Ca_3(PO_4)_2$), having an average diameter of around 2.5 nm. Colloidal Ca fraction is essential in the formation and stabilization of casein micelles [62, 63], influencing the milk coagulation ability [64, 65]. It is known that adding Ca salts to milk decreases rennet clotting time and increases curd firmness [40, 51, 64, 66, 67]. Beyond dairy products, the fortification forms of Ca in other food products are also available, typically as inorganic (e.g., calcium carbonate, calcium hydroxide, calcium chloride, and calcium gluconate) and organic (e.g., calcium citrate, calcium malate, calcium lactate and calcium gluconate).

1.1.3.2 Magnesium

Magnesium (Mg) is the second-most important divalent mineral in milk, with an average concentration of ~4.6 mM. The distribution of Mg in milk is about two-thirds in the serum phase and one-third in the colloidal phase, with ~16% of the soluble Mg being in ionic form (Mg^{2+}) [68, 69]. In dairy production, magnesium is often used as a coagulant in cheese-making. It helps to increase the firmness and texture of the cheese and improve flavor development [70]. In addition to its use as a coagulant in cheese-making, magnesium can also be added to milk-based beverages to increase their magnesium content and provide additional health benefits. The effects of ionic Mg on physicochemical instability of milk protein are very similar to Ca^{2+}, but less pronounced than Ca^{2+} [70]. The binding of magnesium to caseins is pH-dependent, with optimal binding occurring at a pH of around 6.0–7.0. At lower pH levels, binding to caseins is reduced, while at higher pH levels, the protein becomes denatured and loses its ability to bind magnesium.

1.1.3.3 Phosphate

Phosphate is a naturally occurring mineral that can be found in milk. In milk, phosphates play an important role in maintaining the pH of milk and contributing to the buffering capacity of milk [71]. It is present in milk in the form of various compounds, including calcium phosphate, magnesium phosphate, and potassium phosphate. They are commonly used in the dairy industry due to their functional properties, such as their ability to bind calcium ions and stabilize proteins [72, 73]. The most used phosphate in dairy products is sodium phosphate, which is used as an emulsifier, thickener, and pH regulator [52]. Inorganic phosphate is present in milk in various forms, and from a total concentration of about 20 mM, approximately 10 mM are in the colloidal state and 10 mM are diffusible [71]. In the diffusible fraction, inorganic phosphate can be free or combined with calcium to form a calcium

phosphate salt. In the colloidal state, inorganic phosphate and organic phosphate can be combined with calcium to form micellar calcium phosphate, which greatly influences the structure and stability of casein micelles [42, 68].

2. Milk component interactions

The interactions between lactose, milk proteins, and minerals are crucial to produce various dairy ingredients and products with unique textures, stabilities, flavors, and nutritional profiles. The extent of these interactions is a function of many processing and storage parameters including the composition of the system (**Figure 1**). Interactions affect the structure of each component and thereby exert both positive and negative effects of the final product. Thus, understanding these interactions helps the dairy industry create wide range of liquid and solid-state products.

2.1 Lactose-protein interactions

Lactose-protein interactions are fundamental in determining the structure, stability, and functionality of dairy systems. These interactions predominantly occur through hydrophobic interactions, hydrogen bonding, and electrostatic forces [74]. Hydrophobic interactions occur between the nonpolar regions of proteins and lactose molecules, influencing the solubility and stability of the proteins and affecting the texture and viscosity of liquid-state dairy products. In addition, the hydroxyl (OH) groups of lactose can form hydrogen bonds with the amine and carboxyl groups of milk proteins, stabilizing the proteins and affecting their interactions, which influences the overall texture of the products. Electrostatic bonding happens between the positively and negatively charged groups of the molecules. This interaction can be direct or indirect, with lactose complexing with charged groups of proteins. These types of interactions or complexations can lead to changes in protein solubility,

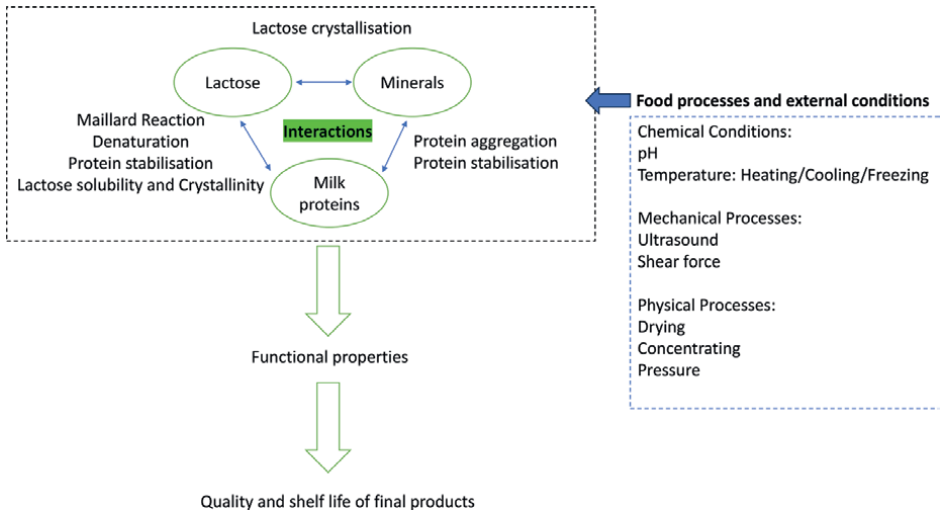

Figure 1.
Schematic representation of the interactions between lactose, milk proteins, and minerals in dairy systems, and how these interactions are influenced by various processing and storage conditions.

stability, and even aggregation within the product. In some instances, excessive hydrogen and electrostatic interactions can lead to texture issues, while balanced hydrophobic interactions can result in desirable textural attributes in some dairy products.

During processing and storage of milk protein products, lactose might be involved in various chemical modifications such as early Maillard reaction or physical modifications such as lactose crystallization where lactose interacts with itself to induce nucleation and crystal formation [75]. Furthermore, the lactose-protein interaction can vary significantly based on environmental factors such as pH, temperature, and the presence of other solutes. In addition, processing conditions, time, and processing methods, such as thermal or non-thermal processing, affect these interactions, thereby changing the ultimate final product consequences. The state of lactose (crystalline or amorphous) and the specific type of proteins involved (e.g., casein, whey proteins) are additional variables that define these interactions. In practical applications, understanding lactose-protein interactions is essential for optimizing the processing and storage of dairy products.

2.1.1 Maillard reaction

Heat treatment has been a cornerstone technology in the food and dairy industries, primarily employed to sterilize and prolong the shelf life of milk products [76–78]. The Maillard reaction, also known as nonenzymatic glycation, is a complex process that occurs between amino acids and reducing sugars during heat treatment. It impacts product quality by causing undesirable flavor, texture, and color changes, nutritional losses, and potentially harmful compounds such as melanoidins [25, 79, 80]. In the case of milk, lactose reacts with the free amino acid side chains of milk proteins (mainly ε-amino groups of lysine residues) to proceed to early, intermediate, and advanced stages of Maillard reaction and forms enormous kinds of Maillard reaction products [25, 81]. The rate of Maillard reaction is affected by temperature, time, water activity (a_w), pH, active reactants, and the type of ratio of reducing sugar to lysine in dairy systems [82]. The Maillard reaction can take place during heat processing of dairy products with a low relative humidity, such as spray drying of milk, and during the processing of liquid products, including sterilization/pasteurization of beverages and liquid foods. Notably, once initiated during the processing phase, the Maillard reaction continues to evolve, affecting the product throughout its shelf life [5, 83].

It is well known that the Maillard reaction is closely associated with the conditions of heat processing, such as temperature and duration of heating [80, 84]. This relationship is attributed to the fact that varying levels of heat can modify the glycation processes and the binding sites of glycoproteins in bovine milk. In a recent study by Zhang et al. [80], raw milk samples heated at 75, 90, 105, 120, or 135°C for durations of 5, 15, or 30s showed increased levels of fluorescence intensity, furosine, lactulose, and 5-hydroxymethylfurfural, indicating a positive correlation with heating temperature and duration. Besides, the number of glycated proteins in milk heated at 135°C for 5 seconds rose from 14 to 49, and binding sites increased from 47 to 166 compared to raw milk. In the Maillard reaction of proteins and peptides, the amino groups of lysine and arginine residues exhibit varying reactivity levels. While lysine stands out as one of the most reactive amino groups, the arginine residue, in comparison, demonstrates considerably lower reactivity [24]. Additionally, lysine residues in caseins seem to be more reactive than in serum proteins, while κ-casein

seems to be the most reactive casein [85]. In a recent study by Nielsen et al. [86], the effect of lactose on the Maillard reaction was explored in six milk protein mixtures, including pure whey protein isolate (WPI), pure casein micellar isolate (MCI), MCI + WPI combination with and without lactose (4.7% w/v), under heat treatment at 121°C for 0, 15, or 30 minutes. They discovered that the initiation of Maillard reaction is highly influenced by the presence of lactose. Notably, the concentration of furosine (a marker of the early-stage Maillard reaction) was significantly ($P < 0.05$) higher in MCI compared to WPI samples before heating, which was likely due to the higher content of lactose in MCI as compared with the level in WPI. Upon heating MCI with lactose for 15 or 30 min, the concentration of furosine increased from an initial 0.015 to 0.112 and 0.109 mol, respectively. A parallel increase in furosine levels was observed in WPI and the MCI/WPI combination when heated in the presence of lactose. Similarly, Paul et al. [87] found that lactose content which is crucial for the level of initial process induced browning of the powder, while additional browning reaction that occurs during storage is mostly independent of lactose content but more by proteins already involved in advanced Maillard reaction steps prior to storage [87]. As a result, they suggested that the levels of lactosylated proteins in the fresh product should be carefully monitored as they could evolve later into melanoidins during storage.

In addition to temperature, the rate of deteriorative reactions and storage stability are related to water activity and water content of dairy systems [75, 88, 89]. Miao and Roos [88] found that the crystallization of lactose enhances Maillard reaction, due in part of the release of water. In addition to this, they also noted that the type of crystals formed plays a critical role. The α-lactose monohydrate is the most commercial solid state, and it has the most stable crystalline form, which are harder, more stable, and also being less hygroscopic, while β-anhydrous crystals have greater hygroscopicity and a much higher solubility [3, 90]. Amorphous lactose is very hygroscopic, and under stressful storage conditions, such as high relative humidity and/or increased temperatures, the metastable amorphous lactose will proceed through an irreversible transition to form stable crystalline forms [91]. Furthermore, the crystallization of amorphous lactose to α-lactose monohydrate releases water. When this occurs in a closed environment (e.g., a bag or can of powder), the relative humidity increases, further promoting reactions that are enhanced at a higher water activity (a_w) [5]. In a study by Ceylan Sahin et al. [89], different water activity was found in spray-dried cheese powder samples stored using three different types of packaging materials for 12 months. Samples with higher water activity were found to have a greater extent of Maillard reaction. Linear correlations were detected between water activity and non-enzymatic browning ($P < 0.01$).

Numerous studies demonstrated that the rate of Maillard reaction increases when the water activity values exceed 0.3 [92, 93]. Morgan et al. [75] found that high temperatures and increased water activity increase Maillard reaction, although lactose crystallization in the system was not directly influenced by the Maillard reaction. Similarly, Yan et al., [94] investigated the relationship between Maillard reaction and lactose crystallization at different water activities. They found that, with the increase in a_w, whey protein-lactose model formed more browning and crystallization products than casein-whey-lactose model, indicating that the presence of micelle macromolecules and the interaction between casein and whey proteins limited the browning and crystallization in casein-whey-lactose models [94].

Moisture increase in dairy products also results in changes in glass transition temperature (Tg). Acevedo et al. [95] found Maillard reaction progresses extremely

slow at temperature below Tg and increases with water content at low relative humidity (RH). Glass transition affects the mobility of the molecules in the spray-dried powders. When the materials are in a glassy state, the molecular mobility is somewhat limited, while in the rubbery state, greater molecular mobility was observed [96–98]. Different molecular mobilities may affect the reaction kinetics of the Maillard reaction. An increase in the reaction rate of Maillard reaction has been observed when the temperature is above the glass transition temperature, and the glass transition temperature of the sample (Tg) is depressed significantly due to water production via Maillard reaction [96–98]. They concluded that this behavior is diffusion-controlled and favored by water plasticization.

Furthermore, Maillard reaction is governed by solid water interactions and structural changes within the system. Amorphous materials undergo structural changes at Tg, which is a function of relative proportion of glass-forming components and water content. Increased water content leads to matrix plasticization, a decrease in Tg, and various chemical and physical changes. These factors can cause the matrix to collapse, affecting the Maillard reaction. So, they highlighted the importance of the system structure. In a study by Miao and Roos [99], a critical value of difference between the particle and its glass transition temperature (T-Tg) was observed. When the temperature differences were above this critical value, a significant increase in the rate of Maillard reactions was observed. At lower temperatures close to Tg, the Maillard reaction rate was low and more water content dependent [99]. A later study by Gómez-Narváez et al. [1] on whey powders also suggested that lactose in the rubbery-like state promotes Maillard reactions. During the spray-drying process, the moisture contents of the particles change rapidly, and thus the glass transition temperatures of the particles also change [1]. The results of Miao and Roos [99] suggested that the change in the glass transition temperature during the spray drying process is also an important factor that needs to be considered when studying Maillard reactions in spray dryers.

The Maillard reaction can influence the solubility of powders. In a related study, Le et al. [81] investigated the protein changes in relation to solubility, Maillard reaction, and protein cross-linking of whole milk power (WMP), skim milk power (SMP), and whey protein concentrate (WPC) when stored at different relative humidities (RHs). They found that an increase in certain Maillard reaction indicators (furosine) correlated with a drop in solubility for MPC, WMP, and SMP, a pattern absent in WPC. The amount of furosine in WMP increased from around 159 mg/100 g of protein to almost 3000 mg/100 g of protein after 12 weeks of storage at 66% RH. This is about twice the amount reached in MPC power under the same conditions. This could be explained by the higher lactose/protein ratio in WMP [81].

The physical and functional properties of powders are affected by the degree of Maillard reaction by enhancing protein interactions and aggregation. Maillard reaction can induce specific protein cross-linking (e.g., GOLD-glyoxal lysine dimer, MOLD-methyglyoxal lysine dimer) [100] through heat and storage, leading to aggregation [81, 101]. Covalent cross-links include intermolecular disulphide bridges, lysinoalanine (LAL) and lanthionine (LAN) [21, 102]. The amount and ratio of covalent/non-covalent aggregation might change when heating in the presence of saccharides, due to the Millard reaction. Supporting this hypothesis, Fan et al. [74] verified that the Maillard reaction promotes protein cross-linking and, in turn, is influenced by protein cross-linking. Specifically, they found that the Maillard reaction was slower when the degree of protein cross-linking was greater in modified milk protein concentrate powders.

2.1.1.1 Glycation

The Maillard reaction is also known to lead to protein glycation, a process in which proteins become modified by the reaction of their amino groups with reducing sugars [103], and thus significantly influences the physicochemical and functional attributes of proteins such as solubility, hydrophobicity, and interactions within the food matrix. The dynamics of the glycation process are determined by various parameters including the nature of the protein and saccharide involved, as well as the environmental conditions such as temperature and pH [104, 105]. An interesting observation by Milkovska-Stamenova and Hoffmann [83] reveals that lactose-free milk exhibits higher glycation levels compared to regular milk. This is attributed to the enhanced reactivity of monosaccharides like D-glucose and D-galactose relative to the disaccharide lactose. Furthermore, the degree of glycation in whey proteins escalates notably when UHT milk is stored at elevated temperatures (28°C and 40°C) as opposed to cooler conditions (4°C), as demonstrated by Holland et al. [104]. This trend is consistent with findings by Guyomarc'h et al. [106], where β-lactoglobulin (β-LG) and β-casein (β-CN) in skim milk powder showed increased glycation at storage temperatures above 4°C. Storage duration is another pivotal factor impacting protein glycation levels in milk products. For instance, Rauh et al. [107] documented an increase in the glycation level of β-lactoglobulin, α-lactalbumin, and caseins in UHT milk after a six-month storage period at room temperature, a discovery made possible through liquid chromatography-mass spectrometry (LC-MS) analysis. Given these insights, it is advisable to store dried milk and whey solids under dry and cool conditions and to practice stock rotation to mitigate browning and other undesirable changes during storage.

2.1.1.2 Lactosylation

Lactosylation is a specific type of glycation reaction that occurs between lactose and proteins or amino acids, and it is an integral component of the Maillard reaction. The occurrence of lactosylation may not cause browning, but can lessen the nutritional quality of milk due to the blockage of lysine, which is no longer available for digestion [92]. This reaction is mainly induced by heat treatment. Nevertheless, some authors have demonstrated that lactosylation also occurs during storage of milk powder [106]. Whey proteins, in particular, undergo lactosylation within a temperature range of 25 to 45°C, with the rate intensifying and peaking at around 65°C. Interestingly, lactosylation progresses more rapidly in a dry state than in a liquid state at elevated temperatures, binding typically 4 to 11 lactose molecules to each protein, although studies have reported varying numbers of lactose molecules per protein [6, 75, 108].

Environmental conditions such as water activity and pH remarkably influence lactosylation. Optimal water activity ranges between 0.4 and 0.8, facilitating the reaction by enhancing component mobility. Moreover, pH levels significantly dictate the extent of lactosylation; for instance, lower pH levels decrease reactive unprotonated amine groups, whereas higher pH levels amplify protein negative charge, leading to increased electrostatic repulsions and, consequently, heightened lactosylation [109]. The rate of lactosylation escalates further with a rise in temperature and relative humidity. However, the presence of the Maillard reaction over time consumes lactosylated proteins, thereby reducing their prevalence in the final product [109].

Lactosylation also leads to the formation of reactive amino acid residues, instigating intra- and intermolecular crosslinks and thereby affecting the protein structure [108]. The interaction between proteins and lactose, and the subsequent Maillard reaction, can cause a loss of protein tertiary structure, inducing steric stress and prompting protein unfolding [86]. In some cases, lactosylation results in a shift in protein structure, such as the β-sheet structure of β-lactoglobulin (BLG), suggesting the formation of hydrogen bonds between BLG and lactose, especially at low relative humidities [7].

The presence of lactose can modify the structure and functionality of milk proteins, leading to changes in properties such as solubility, viscosity, and heat stability [5, 19, 110]. Conversely, milk proteins can also influence the crystallization of lactose in dairy products, thereby affecting properties such as texture and shelf life [111–113]. Thus, knowledge of the mechanisms occurring in protein-lactose systems is important to develop desirable properties in dairy products. In the case of dairy powders, the state of lactose significantly impacts shelf life, particularly in the meta-stable state in whey powders produced by spray-drying or freeze-drying. Under harsh storage conditions, these powders may experience a glass transition zone of lactose, leading to increased molecular mobility and subsequent physiochemical changes like lactose crystallization, particle caking, and lipid oxidation [114–116]. Therefore, controlling the amorphous phase of lactose or managing the crystallization process becomes a pivotal consideration for the dairy industry to mitigate issues like caking, especially in high-temperature and high-humidity environments [117].

2.1.2 Denaturation, coagulation, and protein stabilization

Lactose significantly influences the conformation and aggregation of milk proteins, playing a key role in protein stabilization [118]. Research focusing on the thermal stabilities of whey proteins has demonstrated that lactose, like other sugars, can stabilize protein aggregation under thermal stress [119, 120]. This stabilization effect is further supported by findings showing varying adhesive strengths of lactose in whey deposits on surfaces [121]. Differential scanning calorimetry (DSC) has been employed to illustrate how lactose presence elevates the thermal denaturation temperature (Td) and denaturation enthalpy of proteins such as β-lactoglobulin (β-Lg) and Bovine Serum Albumin (BSA) [75, 81, 122].

The stabilization of protein conformation by lactose is often discussed in terms of preferential hydration, where lactose is excluded from the nonpolar surfaces of proteins, contributing to stability by maintaining or enhancing protein hydration [27, 48, 123]. In dry states, lactose stabilizes proteins by forming hydrogen bonds with protein molecules, replacing those between water and the protein, thereby preserving the protein's native conformation [124, 125]. Additionally, lactose can form amorphous matrices that encapsulate protein molecules, inhibiting structural changes by progressively removing water [126, 127]. This concept of stabilizing proteins within an amorphous sugar matrix is supported (**Figure 2**) by Buera et al. [128].

Lactose also binds to proteins, maintaining their colloidal stability. The physical state of the sample, such as the formation of a glassy matrix by amorphous lactose, plays a crucial role in this process [129]. Hajihashemi et al. [7] emphasized that saccharides, including lactose, protect protein structure through mechanisms like the creation of an amorphous sugar network, which reduces protein mobility and reactivity, or by diluting proteins in a solid state to reduce intermolecular contact.

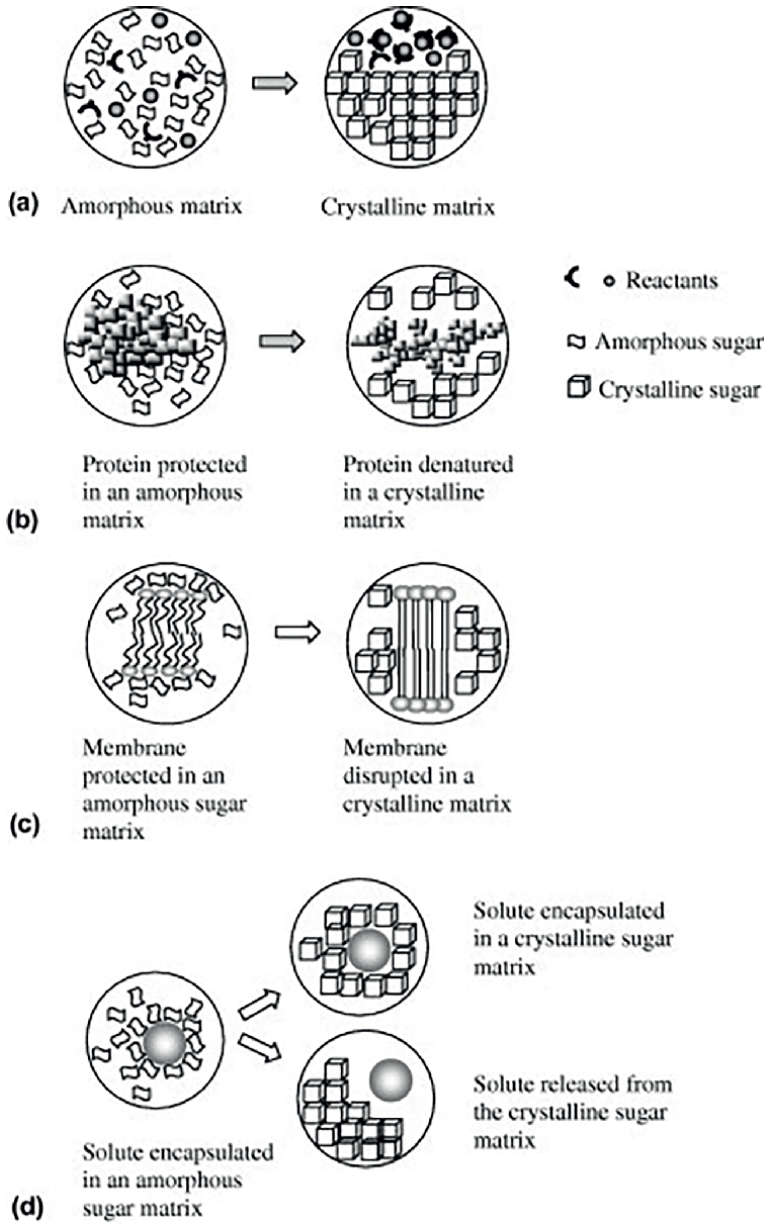

Figure 2.
Schematic interpretation of the effect of sugar crystallization on (a) the acceleration of chemical reactions, (b) protein denaturation, (b) the membrane integrity, and (d) and release of encapsulated compounds. Reprinted with permission from [128].

Furthermore, lactose's hydrophilicity can disrupt the direct interaction of caseins, affecting the protein network [130]. The impact of residual water content on protein stability varies depending on the type of protein; for example, whey proteins remain unaffected by moisture changes, whereas casein proteins are more sensitive to hydrophilic alterations [131].

2.1.3 Lactose solubility and crystallinity

The solubility and crystallinity of lactose are significantly impacted by its molecular interactions with whey proteins, primarily through hydrogen bonding. Proteins have been recognized for their ability to delay the crystallization of lactose, as demonstrated in studies by Thomsen et al. [129] and Hajhashemi et al. [123]. These interactions hinder lactose mobility, potentially due to an elevation in the glass transition temperature (Tg) caused by preferential water sorption, as suggested by Maidannyk and Roos [132].

The crystallization process of lactose is influenced by a multitude of factors including relative humidity (RH), duration, water content, and temperature. Hajihashemi et al. [7] noted that the presence of proteins modifies the crystallization behavior of lactose by altering its Tg. This alteration, alongside molecular rearrangements and interactions within the new microenvironment, significantly impacts crystallization dynamics. During lyophilization, water molecules are replaced by lactose in the β-lactoglobulin (BLG) structure. This substitution, coupled with the intermolecular hydrogen bonding between lactose and proteins, results in elongated chain lengths and retards the crystallization of lactose. Seville et al. [133] observed that, during the drying process, hydrophobic proteins tend to migrate through the aqueous phase and accumulate at the powder surface. Conversely, hydrophilic lactose relocates to the core of the particle. Rapid cooling and water removal during this process often leave lactose in a hygroscopic, unstable, amorphous state. Under conditions of low RH and temperature, lactose molecules fail to arrange efficiently, leading to a structure that is more open and porous. Conversely, an increase in temperature and RH enhances molecular mobility and reduces viscosity, causing lactose to transition into a syrup-like supercooled liquid state. Peleg [134] discussed how, at higher moisture levels, lactose is released from the particle surface due to capillary forces and surface tension. This release leads to the merging of lactose with adjacent particles at contact points, forming viscous liquid bridges. These bridges, initially leading to onset sticking and caking issues, can solidify over time to form robust connections, ultimately affecting the flowability and crystallization behavior of lactose.

Furthermore, the type of protein present is important. They found that crystallization was easily induced in heated skim milk powder (SMP) but delayed or even inhibited by the presence of whey protein concentrates (WPC). WPC35 (with 35% protein and 51% lactose) reacted faster than WPC60 (with 60% protein and 23% lactose). Consequently, the influence of the Maillard reaction on crystallization depended on the protein/lactose ratio in the milk systems studied.

2.2 Lactose-minerals interactions

The interaction between lactose and minerals within milk and dairy products is crucial, markedly impacting their physicochemical and structural characteristics. The nature of these interactions is largely determined by the specific type of mineral present, its concentration, and the ambient conditions of the system, particularly the pH and temperature. These variables collectively influence how lactose interacts with minerals, thereby shaping the overall quality, stability, and functionality of dairy products. Understanding these interactions is paramount for optimizing processing techniques and enhancing the nutritional and sensory profiles of dairy products.

2.2.1 Lactose crystallization

Minerals as impurities in lactose solutions would govern lactose crystallization in a direction that was dependent on the concentration and the type of salt present [30, 135]. Minerals can act as nucleating agents, thus accelerating the crystallization of lactose. Furthermore, minerals can sometimes integrate into the crystal lattice of lactose, altering its growth rate and morphology. Some minerals can also block the active sites on growing crystals and inhibit the crystal growth, either by forming complexes with calcium or by reducing the availability of lactose for crystallization.

2.2.1.1 Influence of Ca on lactose crystallization

Calcium is one of the most important minerals in milk and dairy products, and it plays a crucial role in the interaction with lactose. Jelen and Cloture [135] demonstrated that impurity levels greater than 10% with $CaCl_2$ could retard the crystal growth rate of lactose, although can lead to formation of more stable lactose crystals. Conversely, the addition of NaH_2PO_4 in deproteinated whey increased the growth rate of lactose crystals by 30%. Wijayasinghe et al. [30] concluded that the presence of 0.12 and 0.072% (w/w) Ca significantly increased the enthalpy of water evaporation ($P < 0{\cdot}05$) in comparison with pure lactose. The energy required to evaporate water from the pure lactose solution was ~679 J/g, while it increased to 932 and 946.9 J/g in the presence of 0.12 and 0.072% Ca, respectively. Moreover, the enthalpy of water evaporation decreased to 813.7 J/g with the reduction in Ca concentration (0.035%). The presence of Ca affects the behavior of lactose, hindering the removal of water surrounding lactose molecules by forming a strong hydration layer and thus inhibiting the overall crystallization [30, 31, 136]. The divalent Ca^{2+} ion exerts a strong electric field that promotes the structuring of water; thereby, Ca^{2+} can organize the structure of water [37, 137]. Salts have been shown to affect both nucleation and growth stages of lactose crystallization through differential binding to lactose, depending on the radius/charge ratio of the cation present in the salt and its hydrophilic and hydroscopic nature [29]. Ca, with its high radius/charge ratios of cations, has a low ability to bind lactose [29], although it may be incorporated into lactose crystals, hindering the growth rate and crystal size. These structural changes in water molecules can directly affect the solubility of lactose and subsequent supersaturation of the lactose solution, leading to different behavior during crystallization [29]. Additionally, Ca^{2+} ions strongly interact with four to six layers of water molecules via ion-dipole interactions, further restricting the mobility of water molecules [138] and densely packing them in these hydration layers compared to water molecules in pure lactose solutions, resulting in restricted mobilities. Mimouni et al. [113] suggested that the binding of Ca with water could create local supersaturation spots, promoting the production of small crystals and retarding crystal growth. Moreover, the formation of calcium-lactose complexes is facilitated through the interaction of the hydroxyl groups of lactose molecules with the water molecules residing in the hydration layer of calcium. This interaction has been substantiated through the application of Fourier-transform infrared spectroscopy (FTIR). **Figure 3** depicts the intensity of two prominent FTIR peaks, specifically observed around 1022 and 1065 cm^{-1} in lactose solutions containing calcium. These peaks have been demonstrated to exhibit an inverse relationship with the concentration of calcium in the solution [30, 136]. This observation underscores the complex nature of calcium-lactose interactions and their potential influence on the physicochemical properties of dairy products.

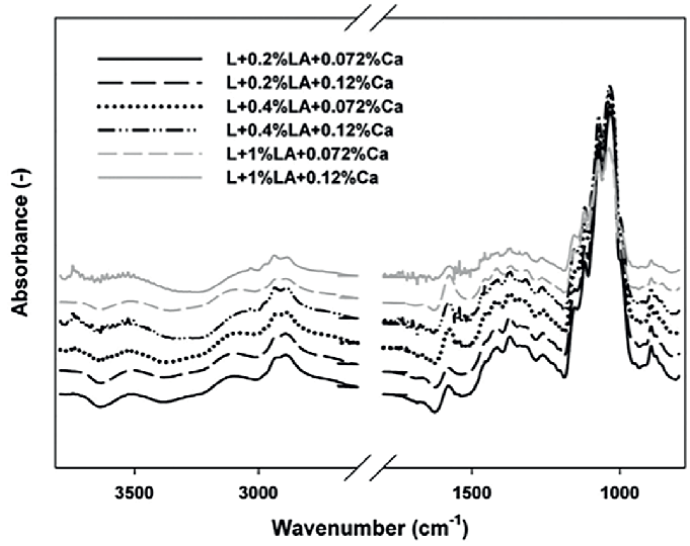

Figure 3.
FTIR spectra for lactose model solutions with the presence of lactic acid (LA) (1% or 0.05% w/w) or Ca (0.12% or 0.035%), control pure lactose (L). Reprinted with permission from [30].

2.2.1.2 Influence of other minerals on lactose crystallization

Other minerals such as magnesium, sodium, potassium, and anions such as phosphate and chlorides, along with trace minerals, can influence lactose crystallization. Magnesium can form less stable complexes with lactose. These complexes may alter the solubility characteristics of lactose and inhibit its crystallization under certain conditions. The formation of magnesium-lactose complexes can reduce the availability of lactose molecules for crystallization, thereby affecting the size, shape, and distribution of lactose crystals in dairy products. Magnesium ions can compete with calcium ions for binding sites in milk. Since calcium is a primary promoter of lactose crystallization, higher concentrations of magnesium can inhibit calcium's ability to facilitate the formation of stable lactose crystals. This competition can lead to slower crystallization kinetics or the formation of less stable crystalline structures [135].

Sodium and potassium influence lactose crystallization to a lesser extent compared to calcium. These ions can change the properties of the medium, such as ionic strength, and thereby alter the solubility characteristics of the lactose solution, influencing crystallization kinetics [136, 138]. Phosphate anions can stabilize calcium-lactose complexes or directly inhibit crystallization in some cases. Chloride ions enhance lactose crystallization kinetics by stabilizing calcium ions or influencing the solubility of lactose. Trace minerals such as iron (Fe), zinc (Zn), and copper (Cu) may act as catalysts or inhibitors based on their concentrations and the presence of specific structures [30]. While calcium is the primary mineral influencing lactose crystallization due to its interactions with lactose and proteins, other minerals also play significant roles depending on their concentrations and chemical interactions in dairy systems. The interactions between lactose and minerals through various mechanisms are of utmost importance for the physico-chemical and functional aspects of dairy products. Therefore, this topic warrants further in-depth research.

2.3 Protein-mineral interactions

The significance of minerals in milk and dairy products is well acknowledged, particularly concerning their role in modulating the thermal stability of milk proteins and milk-based ingredients and their functionality. This modulation is primarily achieved through their influence on protein conformation and structure [139, 140]. The production process of milk protein-based ingredients encompasses several critical stages, each of which can impact calcium partitioning. Calcium ions (Ca^{2+}) are known to induce protein instability by reducing the net superficial charge, which facilitates protein aggregation and enhances hydrophobic interactions. These stages include temperature adjustments (heating and cooling), concentration processes (evaporation and drying treatments), and the separation of milk components, such as through membrane filtration [141–144]. Temperature variations significantly influence protein conformation and stability, as well as the solubility of minerals during both thermal and cold processing. The addition of soluble calcium salts, such as calcium chloride, calcium hydroxide, and calcium gluconate, elevates the concentration of Ca^{2+} ions, potentially leading to protein instability in Ca fortified milk systems. This disruption has profound implications for the solubility, stability, and texture of dairy products, including calcium-fortified milks and various dairy-based ingredients and formulated products. This instability tends to affect whey proteins more than caseins, as documented in several studies [145–149]. The monovalent sodium and potassium ions do not involve in direct interactions, although they influence the ionic strength of the system and the buffering capacity of the milk, leading to protein instability. However, phosphates play a major role in terms of anions. These effects are not only critical during the processing phase but also play a pivotal role in determining the quality and storage stability of the final products [13, 150–153]. The degree to which these changes are permanent or temporary largely depends on the specific conditions of processing and the nature of the changes observed [21]. Thus, understanding the interactions between minerals and milk proteins is vital for producing high-quality dairy products.

2.3.1 Temperature and pH-protein aggregation

Chemical changes occurring within milk during heating also include alterations in the solubility of calcium phosphates, leading to a consequential shift in the pH of milk. This change is part of an equilibrium process involving the formation of insoluble calcium phosphates, as depicted in Eq. (1). Interestingly, most pH-induced denaturation of proteins and alterations in mineral balance are found to be reversible upon cooling the milk [154]. For instance, research has shown that approximately 60% of calcium and 40% of phosphate in the serum phase migrate to the colloidal phase when heated to 90°C for 40 minutes. Upon subsequent cooling to 4°C for 20 hours, between 75 and 90% of the heat-precipitated calcium phosphate is resolubilized. These observations indicate that heating milk to temperatures below 90°C does not significantly impact the composition of calcium phosphate, its dissolution behavior, or the quantity of calcium directly bound to casein, provided the heating is followed by an extended period of cooling [154].

$$3Ca^{2+} + 2HPO_4^{2-} \rightleftharpoons Ca_3\left(PO_4\right)_2 + 2H^+ \quad (1)$$

In the dairy industry, a significant challenge is the formation of milk solid deposits on the surfaces of processing equipment during ultra-high temperature

(UHT) processing of milk products, such as high-protein solutions and creams, a phenomenon known as fouling. Fouling results in product loss, diminished efficiency, and increased energy expenditures [155, 156]. The main culprits for component fouling during thermal processing are the heat-induced destabilization of proteins and the precipitation of calcium phosphate [156, 157]. Moreover, whey proteins in milk, especially β-lactoglobulin (β-LG), are prone to heat-induced denaturation and aggregation. The susceptibility of β-LG stems from two main reasons: its abundance in bovine and most mammalian milk and the presence of a free thiol group that can engage in -SH oxidation or -SH/-S-S- interchange reactions, impacting protein stability and aggregation [21, 158, 159]. In contrast, caseins are more resilient to heat-induced denaturation and aggregation, only affected at temperatures exceeding 100°C. However, calcium phosphate can contribute to fouling deposits even at temperatures above 50°C. Research has shown that calcium ions can promote the heat-induced aggregation of β-LG, with the structure of the aggregates depending on a critical molar ratio of calcium to protein [160–163]. For instance, the addition of calcium to whey protein concentrate (WPC) solutions has been observed to intensify fouling [164]. Similarly, at equivalent protein content and pH, whey protein isolate (WPI) and pure β-LG exhibit less fouling compared to whey, a difference attributed to the higher mineral content of the latter [165]. These observations align with the findings of Petit et al. [166], who investigated the aggregation and fouling behavior of β-LG in relation to [Ca^{2+}]. The study revealed that the overall rate of β-LG unfolding and subsequent aggregation escalated by 1.5 times with increasing [Ca^{2+}], which acted as a catalyst, particularly during the aggregation phase.

Research by Barone et al. [13] indicates that augmenting levels of $CaCl_2$ in infant milk formula reduces the net zeta-potential from −51.1 to −22.1 mV and increases ionic calcium concentration from 1.44 to 6.99 mM. Similarly, Philippe et al. [167] observed that the addition of 4.5 mM $CaCl_2$ to skim milk increased Ca^{2+} levels from 1.56 to 2.86 mM. Moreover, they noted that calcium fortification led to approximately 80% of the mineral associating with the micelle, accompanied by a rise in inorganic phosphate and citrate levels [167]. The increase in Ca^{2+} concentration reduces the surface charge on whey proteins, diminishing the magnitude of electrostatic charge between proteins due to charge shielding. This phenomenon is believed to stem from calcium-mediated bridging between the carboxylic acid groups of aspartic and glutamic acids, fostering the crosslinking of whey protein molecules. Consequently, this process triggers protein aggregation and potentially gel formation [168, 169].

Calcium and phosphate, crucial components of the mineral fraction in milk, significantly influence the coagulation process. The levels of these ions are pivotal in determining the gelation properties of milk. Research has consistently demonstrated that elevated ionic calcium levels enhance the gelation properties of milk [61]. For instance, the introduction of calcium to preheated cow milk at 90°C for 10 minutes notably decreases the gelation time and amplifies the curd-firming rate. This effect arises because calcium addition lowers the pH of the milk, subsequently reducing the electrostatic repulsion between micelles. This reduction facilitates the formation of calcium bridges between casein particles and elevates the level of calcium colloidal phosphate, promoting coagulation. However, there is a threshold to the beneficial effects of calcium addition. Exceeding 10 mM of calcium can adversely affect curd formation. Excessive calcium enhances the positive charges on the micelle surface, leading to increased charge repulsion and, consequently, the formation of weaker gels or the complete inhibition of gelation [170, 171]. Conversely, introducing phosphate into milk results in delayed onset gelation during rennet-induced coagulation.

This delay is attributed to the reduction of the ionic calcium amount, illustrating the delicate balance between minerals and their profound impact on the coagulation properties of milk.

2.3.2 Protein stabilization

Whey proteins, under normal conditions, exhibit considerable physicochemical stability in solution, especially at pH levels distant from their isoelectric point, due to their high charge-to-mass ratio. In the typical pH range of dairy products (6.5–7.0), whey proteins carry a negative charge because of their globular structure, which exposes numerous carboxylic acid residues on their surface [47]. Consequently, whey proteins demonstrate a zeta potential between −35 and − 25 mV, resulting in substantial intermolecular repulsive forces and, hence, a stable physicochemical state, as depicted in **Figure 4**.

The addition of calcium not only facilitates crosslinking between proteins but also induces significant conformational changes in them. This is predominantly due to the closer proximity of proteins caused by calcium-mediated bridges, which displace water molecules between proteins, enhancing hydrophobic interactions

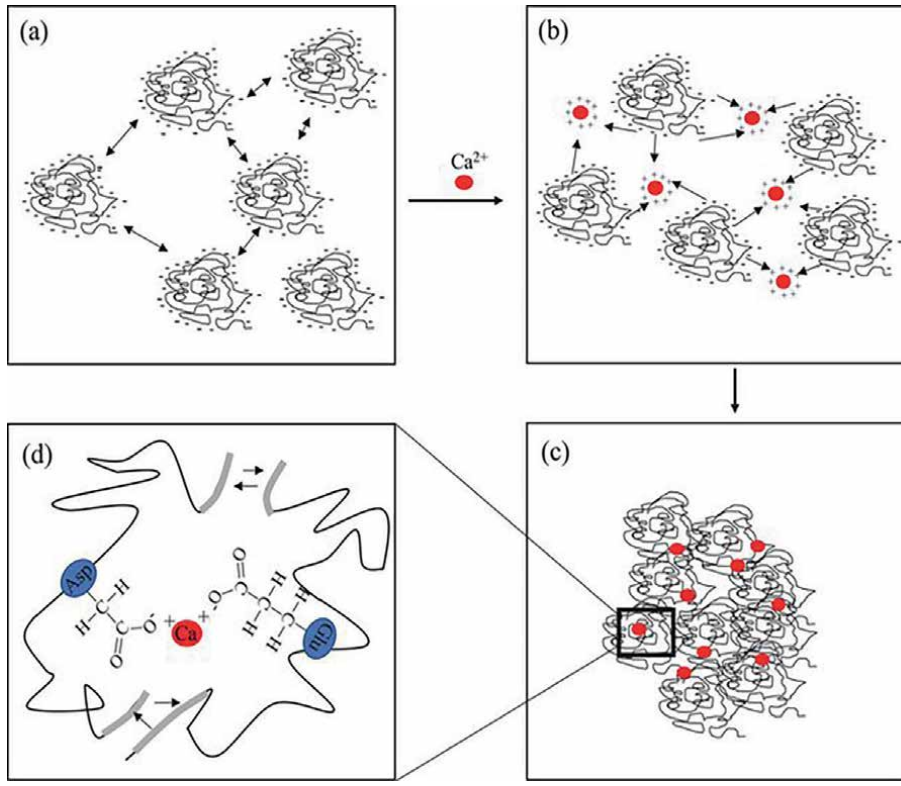

Figure 4.
Schematic illustration of intermolecular attractive forces of proteins mediated by ionic calcium. (a) Innate repulsive forces of whey protein in solution; (b) reduced zeta potential of protein and increased protein intermolecular attractive forces caused by calcium ions (red dots); (c) intermolecular association of proteins mediated by calcium ions; (d) detail of protein-calcium interaction bridges between the carboxylic group of aspartic and glutamic amino acids and attractive interactions between hydrophobic domains of protein (gray) [13]. Reprinted with permission from [54].

within the hydrophobic domains of whey protein. This increased interaction leads to the formation of more thermodynamically stable aggregates, characterized by higher entropy. However, the impact of Ca^{2+} on protein stability can vary. In certain cases, Ca^{2+} enhances the stability of specific proteins, particularly when it binds strongly to intramolecular sites. This effect is well-documented in whey protein α-lactalbumin (α-lac), and to a lesser extent in β-lactoglobulin (β-lg) [172]. α-Lac, in its apo-state (calcium-depleted), exhibits a high affinity for Ca^{2+}, significantly more than in its holo-state (calcium-bound). Binding of Ca^{2+} to apo-α-lac induces conformational changes, increasing the protein's resistance to denaturation under heat treatment by elevating its enthalpy level [173]. This property has been leveraged in the production of value-added whey protein concentrates, enriched in α-lac, which are extensively used in the formulation of nutritional dairy-based products, such as infant formula. Furthermore, the heat capacity of α-lac shows a linear correlation with increasing Ca^{2+} concentrations ranging from 0 to 10 mM, with corresponding ΔH (kJ/mol) values ranging from 217 to 313 [174]. Consequently, the denaturation temperature of α-lac is observed to be a function of the logarithm of $[Ca^{2+}]$. On the other hand, recent studies indicate that calcium can reduce the activation energy and enthalpy required for protein unfolding and denaturation, leading to protein destabilization at lower temperatures [166]. Kaushik et al. [175] found that the thermal stability of Ca-enriched milk (500 ml/L Ca) using various calcium salts (such as Ca-chloride, Ca-acetate, Ca-hydroxide, and Ca-citrate) was lower compared to non-Ca-fortified milk, except for Ca-citrate, which exhibited better heat stability. However, the use of Ca-citrate was associated with increased viscosity, presenting a potential disadvantage in certain applications [175].

3. Conclusions

In conclusion, the interactions between milk proteins, lactose, and minerals in dairy products are complex and multifaceted. A comprehensive understanding of these interactions is crucial for the design, formulation, and processing of dairy products to achieve desirable outcomes. Among these interactions, protein-protein, protein-lactose, lactose-mineral, and protein-mineral interactions are particularly significant. Protein-protein interactions can lead to aggregation, affecting texture and solubility, while protein-lactose interactions, such as the Maillard reaction, can impact flavor and nutritional quality. Lactose-mineral interactions depend on mineral type and concentration and can influence solubility and crystallization patterns. Furthermore, protein-mineral interactions, especially with calcium (Ca), can destabilize proteins by reducing surface charge and enhancing hydrophobic interactions in a concentration- and protein type-dependent way. These interactions occur through various mechanisms, including electrostatic interactions, hydrogen bonding, hydrophobic bonding, covalent linking, and complexation. These interactions are influenced by various intrinsic factors (e.g., type of protein, lactose forms, and calcium salt chemistry), as well as extrinsic factors (e.g., pH, moisture, temperature, ionic strength, pressure, and shear force). To date, progress has been made in understanding interactions between protein, lactose, minerals, and other components in different dairy systems. However, more research is needed to thoroughly investigate ways to maximize the total nutritional value and product performance while avoiding physicochemical destabilization. Future research should focus on further understanding the mechanisms behind these interactions and developing new techniques and

technologies to improve and optimize the quality, functional properties, and nutritional value of dairy products. Most of the above research is based on the interaction between two components, a better understanding of how each of these components present in various properties interacts with each other is vital in controlling the quality of dairy products. In addition, advanced processing technologies need to be explored further to understand any deviations in these interactions compared to the normal processing methods currently employed in the industry. By gaining a comprehensive understanding of these complex interactions, a wide range of value-added products that meet consumer demands for nutritious products could be produced in a sustainable manner.

Conflict of interest

The authors declare no conflict of interest.

Author details

Yuanyuan Zhao[1], Juhi Saxena[2], Tuyen Truong[1,3] and Jayani Chandrapala[1*]

1 School of Science, RMIT University, Bundoora, Australia

2 Spraying Systems Co., PathoSans, Truganina, Australia

3 School of Science, Engineering and Technology, RMIT Vietnam, Ho Chi Minh City, Vietnam

*Address all correspondence to: jayani.chandrapala@rmit.edu.au

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Chapter 5

Formulation of Milk Protein-Derived Dipeptidyl Peptidase-IV Inhibitory Peptides Rich Dietary Supplement: Opportunities and Challenges

Bhimesh Ashritha, Malladevanahalli Huchegowda Sathish Kumar, Priyanka Singh Rao and Channahalli Thammegowda Manoj Kumar

Abstract

Sedentary lifestyle and diet are widely recognised as key risk factors for chronic illnesses like type-2 diabetes. As consumers' food choices are increasingly influenced by nutritional, environmental and health factors, the scientific community focuses on identifying natural bioactive chemicals. Since dairy protein-derived, bioactive peptides can be used as nutraceuticals and medications to treat metabolic disorders with few or no human side effects. In this context, extensive research conducted in the dairy industry over the past two decades has demonstrated that milk proteins, including an abundance of peptides, possess biological features that can mitigate diabetes. Preclinical and clinical research has found some excellent peptides with superior efficacy and safety. Thus, more research on these peptides may lead to clinically beneficial antidiabetics. This chapter gives detailed development of DPP-IV inhibitory-rich dietary supplements.

Keywords: type 2 diabetes, milk proteins, enzymatic hydrolysis, dipeptidyl peptidase-IV inhibitory peptides, dietary supplement

1. Introduction

Noncommunicable diseases (NCD) are the leading causes of death globally, accounting for a larger proportion of deaths that occur prematurely. Therefore, these facts highlight the immediate requirement for worldwide implementation of preventive measures and therapies, as well as the reduction of existing gaps both within and across nations [1]. Dietary supplements are designed to augment or complement the diet and are distinct from traditional meals. In general, if a substance is designed

Enzyme	Substrate	Breakdown products	Place of action	Inhibitor	Mechanism of inhibition
α-Glucosidase	Oligosaccharides, trisaccharides and disaccharides	Glucose and other monosaccharides	brush border of the intestines	α-Glucosidase inhibitor	Inhibit the absorption of carbohydrates from the small intestine
α-Amylase	Starch and glycogen	Dextrins and maltose	Salivary glands and pancreas	α-Amylase inhibitor	Decrease in the amounts of glucose present in the bloodstream by inhibiting the enzyme
DPP-IV	Incretins (GLP and GIP)	Inactive GIP and GLP	Blood stream	DPP-IV inhibitor	Inhibits the inactivation of incretins, which increases the insulin secretion

Table 1.
Inhibition mechanism of α-glucosidase, α-amylase and DPP-IV enzymes.

to treat, diagnose, cure or prevent diseases, it is considered as a drug, regardless of whether it is marketed as a dietary supplement. Supplements are used orally and are available in various formats such as tablets, capsules, soft gels, gel caps, powders, bars, gummies and liquids [2]. Among the many NCDs, diabetes mellitus is a metabolic disorder characterised by hyperglycaemia, or blood glucose levels above the physiological range. Type 2 diabetes mellitus (T2DM) comprises approximately 90% of diagnosed diabetic cases, where patients suffer from inadequate production of insulin by the pancreas and/or resistance to insulin in peripheral tissues. A novel therapeutic method involves the use of α-glucosidase inhibitors [3], amylase inhibitors [4] and dipeptidyl peptidase IV (DPP-IV) inhibitors [5]. The details of the inhibition mechanism of these are presented in **Table 1**.

These inhibitors reduce the absorption of glucose in the intestines and increase the production of insulin, resulting in a hypoglycaemic effect. The administration of synthetic drugs, a commonly used approach, can lead to many undesirable effects including hypoglycaemia, weight gain, exhaustion, diarrhoea and anaemia, among others [6]. Extensive research undertaken in the dairy sector over the past two decades has shown that milk proteins include several peptides with various biological properties, including the potential to alleviate diabetes [7]. Whey protein and casein consumption are linked to increased insulin secretion in the body. Multiple studies have shown that both whey protein and casein have properties that stimulate the release of insulin and lower glucose levels in both healthy individuals and those with type 2 diabetes mellitus [8]. Investigating the potential of bioactive peptides produced from milk proteins offers great potential for the development of nutraceuticals that can effectively address medical conditions without causing adverse effects. These nutraceuticals can offer easy access to intervention and nutritional therapy for patients. In view of the beneficial aspects of milk peptides, the current chapter focuses on the production, commercialisation and challenges of milk-derived DPP-IV inhibitory peptides.

2. Dipeptidyl peptidase IV (DPP-IV) inhibitory peptides

The enzyme dipeptidyl peptidase-IV (DPP-IV) is a serine exopeptidase that cleaves incretins involved in controlling glucose metabolism in the human body [9]. It is a glycoprotein having a molecular weight of 110 kDa commonly found on the surface of cells in many organs such as the kidney, liver, colon, placenta, prostate, skin, lymphocytes and endothelial cells.

Incretins are naturally occurring gut-derived peptides, such as GIP (glucose-dependent insulinotropic polypeptide) and GLP-1 (glucagon-like peptide-1), which are released rapidly in response to the consumption of food. These peptides have a vital role in controlling blood glucose levels by stimulating pancreatic β-cells post-prandially to secrete insulin and inhibiting the release of glucagon by pancreatic cells [10]. However, these peptide hormones are cleaved by DPP-IV at their amino-terminal dipeptide and make them inactive; hence, they have only 1-2 min and 10 min half-life, respectively, for GLP-1 and GIP [11]. The half-life of incretins further reduced to the extent of 30% in people suffering from diabetes [12]. It can be overcome by employing effective DPP-IV inhibitors, and it will help maintain adequate levels of GIP and GLP-1 peptides [13].

The Food and Drug Administration (FDA) has approved several DPP-IV inhibitory drugs such as sitagliptin, linagliptin, vildagliptin, saxagliptin and alogliptin for diabetes treatment. However, they are associated with the side effects on human health such as joint pain and risk of heart failure. Therefore, it has led to exploration of safe alternative DPP-IV inhibitors. Various studies have revealed that hydrolysates of dairy proteins (casein and whey protein fractions) can inhibit DPP-IV activity (**Table 2**). Multiple computational studies have demonstrated the capacity of milk protein hydrolysates to inhibit DPP-IV, as evidenced by the research conducted by the authors in Refs. [18, 27, 28].

Various mechanisms of action for DPP-IV inhibitory peptides have been documented. The types of inhibition mentioned are competitive, non-competitive, uncompetitive and mixed-type modes of inhibition [18]. Primarily, the competitive peptides bind at the active site of the DPP-IV enzyme; thus, it prevents substrate (incretins) from binding with the enzyme. Several peptides have shown their ability to act as competitive inhibitors of DPP-IV, especially proline-containing dipeptides, that is, Ile-Pro. These peptides are highly effective in inhibition of DPP-IV even at lower micromolar range [29]. Notable proline containing DPP-IV inhibitor peptides include Ile-Pro-Ile and Val-Pro-Ala [30]. The amino-terminal XaaPro- or Xaa-Ala-dipeptides are also potential competitive DPP-IV inhibitors which either deactivate or modify functions of the DPP-IV enzyme. These competitive inhibitors play an outstanding role in investigating food-derived DPP-IV inhibitory peptides. The non-competitive, uncompetitive and mixed-type modes of interaction are limited to interactions occurring outside the active sites of DPP-IVs, in comparison with competitive inhibitors [31]. Non-competitive inhibitors can specifically bind to secondary binding sites and make an inactive complex, which lowers DPP-IV's catalytic activity. Peptides that are longer than 13 residues have been demonstrated to function as non-competitive inhibitors by establishing contacts at the dimerisation interface and obstructing the creation of the active dimer of DPP-IV [32]. Recently, a dipeptide called Trp-Val, produced from milk protein, was discovered. This dipeptide acts as a non-competitive inhibitor of DPP-IV. Molecular docking analysis demonstrated that Trp-Val is highly probable to engage with DPP-IV at a secondary binding site located

Source of protein	Enzyme/microorganism used in hydrolysis	Identified peptide sequence	IC_{50} value*	References
β-Lactoglobulin	Trypsin	VAGTWY	174 μM	[14]
Gouda cheese		LPQNIPP	<200 μM	[15]
WPC rich in bovine β-lactoglobulin	Trypsin	IPVAF TPEVDDEALEK IPAVFK	44.7 ± 3.6 mg/ml 319.5 ± 4.0 143.0 ± 1.3	[16]
Whey protein α-Lactalbumin β-Lactoglobulin Lactoferrin Bovine serum albumin Whey protein	Pepsin	Not available	0.036 ± 0.002 mg/ml 1.279 ± 0.100 0.379 ± 0.035 0.513 ± 0.056 0.075 ± 0.006	[17]
Whey protein hydrolysate	Corolase pp	Not available	1.33 ± 0.177 mg/ml	[18]
Bovine whey protein isolate and α-lactalbumin	Pepsin	VLVLDTDYK and WLAHKALCSEKLDQ LAHKALCSEKL TKCEVFRE LCSEKLDQ LKPTPEGDL LKPTPEGDLEIL	424.4 μM 141 165 166 186 45 57	[19]
Milk protein and protein hydrolysates	Trypsin	Not available		[20]
Milk protein	Pepsin and pancreatin	LKTPEGDL LPYPY IPIQY IPI WR	12.5 mg/ml 75 50 12.5 75	[21]
α-Lactalbumin	Alcalase	ELKDLKGY ILDKVGINY		[22]
β-Casein	Flavourzyme	YPVEPFTESQS	3.26 mM	[23]

Source of protein	Enzyme/microorganism used in hydrolysis	Identified peptide sequence	IC_{50} value*	References
Ultrafiltered goat milk	*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarus* subsp. *thermophilus*	INNQFLPYPY	Not available	[24]
Casein	*Lactobacillus helveticus* CICC6024	α_{S1}-Casein – VAPFPE	Not available	[25]
Whey proteins hydrolysates	*Enterococcus faecalis* 2/28	β-Lactoglobulin-LKPTPEGDLEIL, LKPTPEGDLE α-Lactalbumin-LKGYGGVSLPE, ILDKVGINY	Not available	[26]

**IC50 represents the concentration at which a substance exerts half of its maximal inhibitory effect. As reported by the FDA, the IC50 value represents the minimal concentration of a drug that is required for 50% inhibition in vitro. Traditionally, this value is expressed as a molar concentration.*

Table 2.
DPP-IV inhibitory peptides identified in milk.

in close proximity to the active site [33]. In the uncompetitive DPP-IV inhibition mode, inhibitors only bind to the substrate–enzyme complex. This lowers the activity of the enzyme and keeps the substrate away from its active sites. Furthermore, there is a data suggesting that the peptides can operate through a combination of inhibitory mechanisms [34]. This process entails the simultaneous attachment of two inhibitor molecules at distinct locations on the enzyme.

In addition to DPP-IV inhibition, the administration of milk protein or milk protein hydrolysate may provide additional benefits to host such as increase in globulin levels and HDL and reduction in the levels of urea, creatinine and bilirubin [15].

3. Production of milk-derived DPP-IV inhibitory peptides

Food-grade bioactive peptides are produced by either enzymatic hydrolysis or microbial fermentation of milk protein substrate as they have latent in the primary sequence of protein and hydrolysis release the active fragments. The advantage of using fermentation technique is the less cost of bacterial culture to generate the peptides; however, less specificity, long duration of fermentation and additional metabolites production limit its use. The second technique, that is, enzymatic hydrolysis of milk proteins, addresses these disadvantages as it has high specific activity in less hydrolysis duration, making it more suitable for industrial level production. Hydrolysis is followed by a series of purification and identification steps guided by the activity to identify the most effective sequence (**Figure 1**). Additionally, certain studies employed a mixture of these approaches to generate peptides that possess a biological function [35].

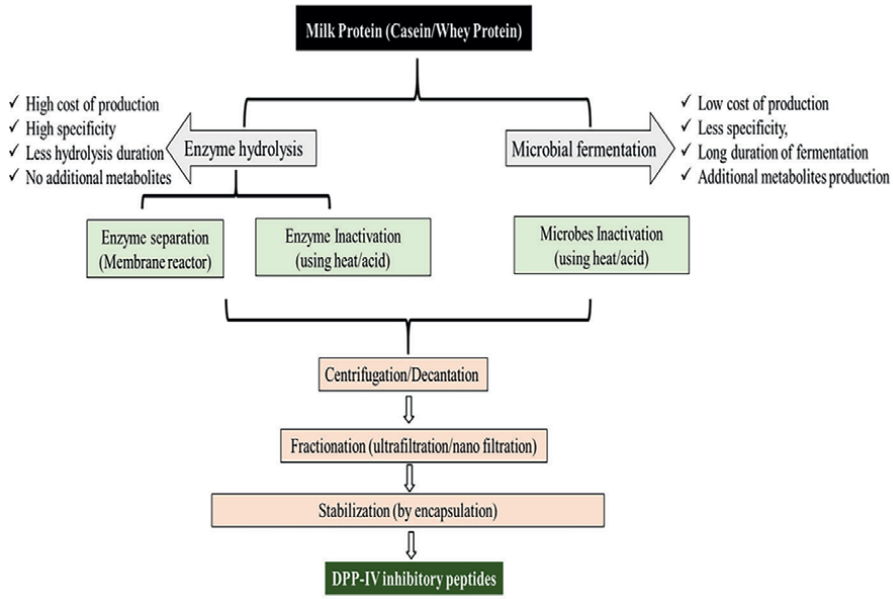

Figure 1.
General flowchart for production of DPP-IV inhibitory peptides from milk protein.

3.1 Enzymatic hydrolysis

Enzymatic hydrolysis is a digestive process that involves breaking peptide bonds between two amino acids. This process consumes one molecule of water for each bond cleaved and produces lower molecular weight compounds such as peptones, peptides and amino acids [36]. Enzymatic hydrolysis necessitates gentle reaction conditions and is characterised by its specificity and controllability. The reaction is simple, requiring the substrate (protein) and the enzyme(s) (protease(s)). The protease determines the reaction conditions, including pH (ranging from 4 to 8) and temperature (ranging from 40 to 60°C). Additionally, parameters such as enzyme/substrate ratio and substrate concentration must also be considered. Milk proteins can be used to generate DPP-IV inhibitory peptides through the use of single or multiple enzymes obtained from plants (such as papain), animals (such as pepsin, trypsin and Corolase PP), or microorganisms (such as alcalase, protame and flavourzyme) [19–21]. On the other hand, the water-soluble extract from natto is a unique example where hydrolysates were formed during food processing without the use of external enzymes. However, the DPP-IV IC50 value (5.35 mg/mL) still appeared to be relatively high [37].

Highly bioactive hydrolysates are typically obtained by employing several experimental methods to attain ideal conditions [22]. The process of enzymatic hydrolysis is often conducted in a reactor that is equipped with a jacket to maintain a consistent temperature. Stirring is employed to achieve uniformity throughout the reaction. Regarding the synthesis on a big scale, certain authors have manufactured protein hydrolysates at a pilot or semi-pilot plant scale. Nevertheless, the utilisation of enzymes can escalate the expenses associated with the procedure, therefore making it more desirable to use economical sources of enzymes.

In addition, the hydrolysis efficiency of a protein hydrolysate is defined by the degree of hydrolysis, which is the proportion of cleaved peptide bonds in the hydrolysate [23]. As the degree of hydrolysis increases, the size of the peptides in the resulting product decreases. A higher degree of hydrolysis following enzymatic digestion suggests improved intestinal absorption and increased accessibility to the active areas of DPP-IV. Thus, hydrolysates that inhibit DPP-IV with the lowest IC_{50} values can be chosen and separated for the purpose of identifying bioactive peptides.

The researchers discovered a new peptide called LDQWLCEKL in bovine whey α-lactalbumin using trypsin hydrolysis. This peptide has a DPP-IV inhibiting activity with an IC50 value of 131 μM. The findings suggest that LDQWLCEKL could be used as a preventive or adjuvant medication for managing type 2 diabetes [27]. In a previous study by [17], it was found that pepsin digestion of β-lactoglobulin resulted in the enrichment of two very effective uncompetitive DPP-IV inhibitory fragments, namely LKPTPEGDL and LKPTPEGDLEIL. The IC_{50} values of these fractions were determined to be 45 and 57 μM, respectively.

3.2 Fermentation

Lactic acid bacteria rely on milk protein as their primary supply of vital and growth-promoting amino acids. Microbial-based proteolysis, specifically by fermentation by lactic acid bacteria (LAB), is considered a cost-effective, environmentally friendly and safe alternative to enzymatic hydrolysis for producing bioactive peptides obtained from whey protein. The key factors in utilising microbial fermentation are the careful selection of appropriate strains for specific proteins, establishing an

optimal temperature conducive to bacterial growth, and effectively managing the duration of the fermentation process. The process of producing biopeptides through microbial fermentation is simple. Initially, the chosen microbe is cultivated until it reaches its exponential growth phase in a suitable medium under the most favourable conditions. Afterwards, the microbes are collected, cleansed and suspended in a sterile solution. They are then introduced into the desired food substance as a starter to initiate the fermentation process and generate biopeptides [36, 38]. The consequences of the fermentation process, specifically the types and quantities of peptides generated, are greatly influenced by the type of microbe, the nature of the food matrix and fermentation circumstances. The presence of diverse microbial proteinases in each strain allows for the production of multifunctional peptides [39]. This leads to the formation of peptide sequences with a broad range of biological functions. Furthermore, microorganisms serve as a cost-effective supply of proteases. Neutrase, Subtilisin, Orientase, Protex 7 L, Protamex 1.5 and proteases from lactic acid bacteria (LAB) are commonly employed in the manufacturing of bioactive peptides [40].

In a study, [41] discovered three antidiabetic peptides obtained through the separation of β-lactoglobulin after a fermentation procedure conducted by *S. thermophilus* RBC06, RBC20 and RBN16. These peptides exhibited different molecular weights. APFPE, a peptide sequence found in α_{S1}-casein (26–30); VPITPTL, a peptide sequence found in α_{S2}-casein (117–123); and LPQNIPP, a peptide sequence found in β-casein (70–76), have demonstrated DPP-IV inhibition. The identical genus of lactic acid bacteria has been employed in the fermentation of whey proteins, revealing a DPP-IV inhibitory capability with an IC_{50} value of approximately 50 μmol/L. Nevertheless, there is a severe lack of research on the release of antidiabetic peptides derived from coculture fermentation with lactic acid bacteria. In a study, [42] produced DPP-IV inhibitory peptides by fermenting milk whey with *Streptococcus thermophilus* SY-102, *Lacticaseibacillus rhamnosus GG* and a combination of both bacteria. The fermentation process involved incubating 10% whey solutions at 42°C and collecting samples every 3 hours. The findings of this investigation demonstrate that *L. rhamnosus GG*, both in monoculture and in a coculture system with S. thermophilus SY-102, produces peptides that exhibit a significant level of DPP-IV inhibitory action above 50%.

3.3 Fractionation, purification and identification

Peptidomics is a technology based on omics that emerged in the early twenty-first century as an extension of the shot-gun proteomics method. The objective of peptidomics is to ascertain the variety of peptides found in a biological sample, encompassing food sources. The peptidomics workflow comprises the processes of peptide extraction and purification, separation, and subsequent bioinformatics analysis for the purpose of identifying and quantifying peptides [43]. Peptides generally consist of 2–20 amino acid residues, and their biological activity is determined by the specific combinations and order of amino acids in their structure.

Recent research has indicated that after being released by enzymatic hydrolysis, the majority of peptide sequences contained within dietary proteins provide bioactive characteristics. The process of concentrating and purifying bioactive fractions from hydrolysates is a crucial step in industrial use. After acquiring a bioactive protein hydrolysate, various technologies can be used to separate peptides depending on their distinct physicochemical features, such as molecular weight, polarity or charge. The extract underwent screening to identify its unique bioactivity, followed by fractionation

utilising a bioassay-driven fractionation method. Ultimately, a single bioactive peptide was obtained through purification. Characterisation methods of the DPP-IV inhibitory peptides are mentioned in **Table 3** briefly. The primary methodologies utilised for fractionation are chromatography and membranes [35]. Chromatography techniques encompass membrane filtering systems, gel or size-exclusion chromatography, capillary electrophoresis (CE), ion-exchange column chromatography and reverse-phase high-performance liquid chromatography (RP-HPLC).

Characterisation method	Type	Advantages	Disadvantages
Fractionation and Purification	Membrane filtration (ultrafiltration)	• Inexpensive, simple and scalable • Preserves the physiological properties of the peptide	• Minimal specificity • Interactions can occur between peptides • Poor reproducibility • Difficulty in obtaining pure peptides • Large sample volume is required
	Chromatographic separation (size-exclusion chromatography, RP-HPLC, ion-exchange column chromatography, CE)	• Higher resolution • Generation of high-quality (very pure) products	• Time-consuming • Expensive, raising the price of commercialising bioactive peptides
Identification	LC-MS	• High sensitivity and accuracy	• chromatographic separation of peptides is quiet difficult
	MALDI-TOF MS	• Resilience • Tolerance for the presence of impurities	• Sample preparation is variable • Different preferential peaks for identical samples • High cost of industrialisation • High cost of industrialisation
	UHPLC–ESI–MS/MS	• Rapid separation capabilities • Low solvent usage • High capacity, resolution, sensitivity, specificity and selectivity	• High cost of industrialisation
	Bioinformatics	• Guide protein precursor selection • discovery of novel peptides • reliability • relatively cost-effective	• Not guaranteed that *in silico* peptides can be reproduced experimentally • Does not consider other peptide sequences within the "in silico hydrolysates" other than that exist in databases

Table 3.
Characteristics of different methods involved in DPP-IV inhibitory peptide generation from milk protein.

The subsequent procedure involves the utilisation of MALDI-TOF MS (matrix-assisted laser desorption ionisation-time of flight mass spectrometry), Ultra-high-performance liquid chromatography (UHPLC) and Liquid chromatography coupled to mass spectrometry (LC-MS) for identification purposes. Additionally, bioinformatics analysis, including peptide sequencing database, semi-quantitative analysis database and bioactive peptides database, will be employed to confirm the functionality of the peptides. Finally, assays will be conducted using chemically synthesised peptides to determine the actual bioactivity of the identified peptides [44].

A significant number of the peptides that have been examined and documented for their DPP-IV inhibitory action are composed of two amino acids known as dipeptides. Aside from the 70 dipeptides that possess DPP-IV inhibitory action, there are 43 more peptides, ranging from three to ten amino acids in length, which can be found in widely ingested proteins like milk and soy. These peptides have demonstrated the ability to block the DPP-IV enzyme. The peptides exhibit a potency comparable to the peptides found in protein hydrolysates, with IC_{50} values ranging from 4 to >20,000 μM. The tripeptide IPI, also referred to as diprotin A, is a highly recognised DPP-IV inhibitor. It is derived from the sequence of κ-casein and is currently the most powerful peptide inhibitor known, with an IC50 value of approximately 4 μM. While diprotin A is more effective, the peptides WR, IPIQY and WCKDDQNPHS found in lactoferrin, κ-casein and α-lactalbumin, respectively, are some of the most powerful DPP-IV inhibitors produced from dietary proteins, as described by [45].

4. Offering milk-derived DPP-IV inhibitory peptides to manage diabetes: commercially feasible solutions

As discussed in previous sections, DPP-IV inhibitory peptides can be produced from milk through enzymatic hydrolysis or fermentation. However, the efficacy of these peptides varies from a few micro-moles to several mili-moles in terms of their IC_{50} value [14, 16]. Further, production, purification and isolation of the specific DPP-IV inhibitory peptide from milk proteins is laborious, time-consuming and may yield very low quantity of the specific peptide. Moreover, milk-derived DPP-IV inhibitory peptides cannot replace the commercially available synthetic DPP-IV inhibitory drugs (glyptins) as these drugs exhibit IC_{50} value in a few nanomoles [46] on a one-to-one basis. Therefore, producing DPP-IV inhibitory peptide-rich hydrolysates from milk proteins and offering it as a dietary supplement could be the viable solution at this moment. This supplement could be used as a monotherapy in people suffering from prediabetes and as adjunct therapy in diabetes [47]. Biological complementary therapies, categorised as dietary supplements, are exempt from the rigorous clearance process mandated for medicines under the Dietary Supplement Health and Education Act of 1994 [2]. Hence, dietary supplements are exempt from the requirement to demonstrate safety and efficacy in order to be sold.

4.1 Formulation of DPP-IV inhibitory peptides rich milk protein hydrolysate-based dietary supplement

The dietary supplements are mostly in powder form and usually have a longer shelf life (6 months or more) and can be stored at ambient temperature [48]. In order to formulate a DPP-IV inhibitory peptide-rich milk protein hydrolysate-based dietary supplement, it is essential to consider the following aspects.

i. *The composition of the matrix*

DPP-IV inhibitory peptide-rich milk protein hydrolysate cannot be dried alone as it mostly contains low molecular weight peptides. Hence, the hydrolysate as a whole exhibits low glass transition temperature (Tg). Consequently, the resultant powder has hygroscopicity and thermoplasticity [49]. In order to overcome these difficulties, other constituents especially high molecular weight polysaccharides, are mixed with the protein hydrolysates before spray drying. The spray-drying matrix may also have fat in it, especially when peptides are encapsulated in a double-emulsion matrix [50]. The presence of fat in food affects the structure of proteins by reducing their solubility and increasing the amounts of α-helix and β-sheet structures [51]. It has been demonstrated that changing chemical interactions among molecules might lead to alterations in their bioactivity. These interactions lead to glycation, dimer formation and Amadori product formation during spray drying [52]. These changes could be intra-molecular or inter-molecular depending on pH, temperature and ionic environment. Therefore, proper examination of these interactions is necessary while finalising the matrix material for peptides or protein hydrolysates.

ii. *The glycaemic index of the matrix constituents*

Low glycaemic index (< 55) matrix materials are preferred. Variations in GI values are mostly due to differences in the rate of carbohydrate digestion or absorption within foods [53]. It has been demonstrated that dietary fibre modifies the rate of glucose absorption as well as the pace and degree of starch breakdown [54]. Natural polysaccharides generally have low GI. Gum Arabic, carboxymethyl cellulose, chitosan, cyclodextrin, xanthan gum, pectin and resistant maltodextrin are examples of polysaccharides derived from plants, animals and microbial sources that have low GI and can be used as matrix materials for DPP-IV inhibitory peptides.

iii. *The protection offered by the matrix during gastrointestinal transit*

Polysaccharides have a structure that gives stability during regulated release, low viscosity, high solubility in an aqueous solution and good emulsifying capability [55]. Polysaccharides can also form a thick film around the core, increase the viscosity of the continuous phase and protect the core as it travels through the gastrointestinal tract [56].

iv. *Bitter taste masking ability of the matrix*

Peptide bitterness results from peptide bond hydrolysis and the release of hydrophobic amino acids from protein internal structures [57]. Nevertheless, the bioactive peptides made from various protein sources have varying degrees of bitterness. DPP-IV inhibiotry peptides are often hydrophobic, contain branched chain amino acids and aromatic amino acids and hence, do exhibit bitterness [58]. The bitterness can be masked by encapsulation. Also, encapsulation improves the stability, solubility and bioavailability of bioactive substances that are sensitive to environmental conditions in food products [59].

4.2 Encapsulation of DPP-IV inhibitory peptides

Encapsulation of DPP-IV inhibitory peptides can be done through various methods such as double emulsion with and without Pickering, liposomes, niosomes

and enteric-coated capsules; spray drying is being developed to ensure the safe and efficient delivery of these peptides [60]. Therefore, in recent years, the assessment of microencapsulation of protein hydrolysates has been conducted. Spray drying is the predominant and cost-effective technology used in industry to turn liquid food into a powdered form, among several microencapsulation techniques [61]. Hence, several studies have specifically investigated the impact of spray drying on decreasing the hygroscopicity and bitter flavour of the powders derived from protein hydrolysates. The success of encapsulation is significantly influenced by the choice of wall material, as per studies. Maltodextrin, maltodextrin/β-cyclodextrin and maltodextrin/gum. Gum Arabic has been utilised as a coating material for peptides derived from casein hydrolysate, whey protein hydrolysate and chicken meat protein hydrolysate [62]. An inlet temperature in the range of 160-200°C and an outlet temperature in the range of 60 to 90°C were experimented with peptides and protein hydrolysates [63]. As a prerequisite for bioactive peptides to effectively perform their physiological activities and functions as functional foods or nutraceuticals, it is important for them to have sustained release, good biostability and high bioavailability when encapsulated [64]. This is because the peptides must reach the absorption site in an active state. Guar gum, cyclodextrins and resistant maltodextrin showed potential for encapsulating DPP-IV inhibitory peptides due to their low glycaemic index properties [51].

In a double emulsion system of encapsulation, DPP-IV inhibitory peptide derived from hydrolysed α-lactalbumin of Gir cow milk exhibited roughly four times greater functionality than non-encapsulated, specifically in terms of DPP-IV inhibition activity [46]. A work [26] assessed the ability of α-lactalbumin in non-encapsulated hydrolysate (NEH), freeze-dried encapsulated hydrolysate (FDEH) and emulsified encapsulated hydrolysate in double emulsion (EEH) forms to inhibit DPP-IV in vitro and to serve as an antidiabetic agent in vivo. The percentage of DPP-IV inhibition by the NEH, FDEH and EEH following simulated gastrointestinal digestion was 36 ± 2.28, 54 ± 2.02 and 64 ± 2.02, respectively.

Peptides may denature and alter their functionality after being exposed to spray-drying temperatures [65]. To overcome this effect researchers have explored the electro-spraying method for encapsulation of peptides as it operates at ambient temperature [66]. Although it operates at low temperature, use of specific solvents can induce protein denaturation and loss of bioactivity when expose to longer duration. In addition, exposure to higher electric field strength (application of higher voltage, to the tune of 22-24 kV) also hastens the peptide or protein aggregation which leads to loss of activity [67]. High encapsulation efficiency ranging from 70 to 99% was reported for electro-spraying. Further, compared to mono-axial electro-spraying, co-axial electro-spraying offers better encapsulation efficiency.

4.3 Challenges and regulatory aspects of DPP-IV inhibitory peptides

The challenges encompass several issues such as expensive production costs, undesirable sensory characteristics in final products and alterations in the taste of carrier food products. During the process of milk protein hydrolysis, there is a significant focus on the presence of physiologically active peptides that have health benefits. However, there is also concern about the possibility of cytotoxic or allergic peptides. As a result, it is necessary to conduct a clinical safety evaluation as a mandatory step. Although the bioactive peptide has beneficial properties, its widespread commercialisation has not yet reached its full potential. This could be attributed to the varying allergenicity profiles of different peptides produced using proteolysis

methods, particularly in infants. Currently, there is a lack of a comprehensive guideline for assessing the safety of peptide-like functional meals. As a result, in vivo experiments conducted on rodents are often regarded as the established norm. The primary obstacle in the food sector for producing DPP-IV inhibitory peptides is the significant expense involved in their manufacturing, as these methods are generally not cost-effective [68].

Similarly, there are several challenges in the process of commercialising nutraceuticals and functional foods that contain bioactive peptides. These challenges include (i) difficulties in developing reliable methods to ensure quality, (ii) limited information on how these peptides are absorbed and metabolised in the body and (iii) insufficient clinical evidence to support their effectiveness [17]. Moreover, the hypothetical peptide sequences must be separated from their original proteins in order to become effective. The ultimate determination of the ability of dietary proteins to inhibit DPP-IV is possible only by experimentally evaluating the release of these biologically active peptides through in vitro or in vivo hydrolysis. The entire procedure is quite time-consuming [69]. An essential inquiry about the regulatory status of protein hydrolysates and their fractions depends on whether these products are classified as foods, food ingredients, or dietary supplements and, therefore, subject to particular regulation [70].

Dietary proteins and protein hydrolysates are considered generally recognised as safe (GRAS) in the United States and are permitted in the majority of countries. The majority of countries do not have clear laws regarding the utilisation of amino acids and bioactive peptides as dietary supplements or food additives. In the Netherlands, the official regulations prohibit the addition of single amino acids to foods. However, the Health Council of the Netherlands (1999) provided a favourable recommendation for their usage, but with specific limitations. The following consideration is applicable to hydrolysate fractions and bioactive peptides produced from these hydrolysates. Regulation (EC) No. 1925/2006 (Commission of the European Communities, 2006b) offers a mechanism to address the matter of safety.

4.4 Economics of production with scale

Although there has been a recent increase in the production of commercial products utilising bioactive peptides and hydrolysates, industries do not appear to be at the forefront of this biotechnology industry. In short, despite the numerous scientific studies conducted on the functioning and bioactivity of peptides with hydrolysates from natural sources, only a small fraction of these discoveries have been implemented in the industry. This could be attributed to various factors, such as expensive infrastructural and operational expenses, sensory concerns, limited market acceptance due to manufacturers' claims being uncertain and inadequate return on invested capital, legal and religious complications in certain countries and the products being unaffordable for the general public. The scale of operation has a greater influence on the cost of production. Considering one sieve of dietary supplement between 15 and 20 g per day per person, the requirement for 10% of the population suffering from diabetes and prediabetes would be 1452 MT per day. It is a huge potential market for any industry to capture. If the production scale is huge, all possibility that production cost also favourably low, owing to reduction in enzyme and other raw material costs come down drastically. However, if the scale of operation is small, then production cost will increase and hence difficult to penetrate the market as metformin is commonly available at an affordable price to monitor diabetes & prediabetes. On average,

one can expect a production cost of ₹500-₹1000 ($6-12) per kg of DPP-IV inhibitory peptides-rich hydrolysate production in India.

5. Future perspectives

The focus of research on DPP-IV inhibitory peptides is currently centred on enhancing the potency of hydrolysate proteins and peptides. Nevertheless, the exploration of new peptides derived from food proteins presents many challenges. These include identifying the most effective cost-competitive techniques for isolating and purifying individual peptides from complex mixtures, addressing the limited bioactivity efficiency of peptides, assessing the potential toxicity of peptides on human health and selecting the most suitable cell and animal models for studying antidiabetic properties. Cell cultures and small animal studies are necessary to determine the effectiveness of the current research on the human body and to identify the appropriate dosage of hydrolysate peptides for alleviating diabetes features *in vivo.*

6. Conclusion

Milk protein-derived DPP-IV inhibitory peptides have a better prospectus to offer as dietary supplements to manage prediabetes and diabetes. At this stage, considering the potential so far reported, milk protein-derived DPP-IV inhibitory peptides dietary supplement could be used as a monotherapy to treat prediabetes and as an adjunct therapy for treating diabetes along with other medications; besides, this dietary supplement could reduce the drug load in persons suffering from diabetes. However, there is a need to address the sensory palatability of the product. Further, a couple of complete clinical studies involving human subjects are required to prove the efficacy of this supplement beyond doubt. Although research is taking place at many locations, it could be a matter of time before seeing this product in a market shelf shortly.

Author details

Bhimesh Ashritha, Malladevanahalli Huchegowda Sathish Kumar*,
Priyanka Singh Rao and Channahalli Thammegowda Manoj Kumar
Dairy Processing Section, SRS, ICAR-NDRI, Adugodi, Bengaluru, Karnataka, India

*Address all correspondence to: sathishndri2021@gmail.com

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Chapter 6

Phos-tag SDS-PAGE for the Analysis of Milk Phosphoproteins Involved in Cow's Milk Allergy

Eiji Kinoshita and Emiko Kinoshita-Kikuta

Abstract

Phos-tag functions as a phosphate-binding tag molecule in an aqueous solution under near-physiological conditions. Its affinity for a divalent phosphate ion is 16,000 times greater than that for a monovalent carboxylate ion at neutral pH. We have developed and applied useful techniques for the analysis of phosphoproteins based on Phos-tag. Among these, this chapter presents a phosphate affinity technique for the analysis of phosphoproteins by electrophoresis using Phos-tag. Our electrophoretic method using SDS-PAGE, which is widely used for molecular weight-based separation of proteins, allowed us to separate and detect phosphoproteins and non-phospho counterparts on an identical SDS-PAGE gel. Here we describe the resolving power of Phos-tag SDS-PAGE for the separation and detection of milk phosphoproteins, α-casein and β-casein, as typical protein samples. This technique would have a major impact not only on the analysis of milk phosphoproteins involved in cow's milk allergy but also on the analysis of all food phosphoproteins.

Keywords: Phos-tag, affinity electrophoresis, SDS-PAGE, phosphoprotein, casein, cow's milk allergy

1. Introduction

Phos-tag, a functional binuclear metal complex of 1,3-bis[bis(pyridin-2-ylmethyl)-amino]propan-2-olato, reversibly captures a divalent phosphate ion in aqueous solution under near physiological conditions [1]. Its binding capacity for the phosphate dianion is 16,000 times stronger than that for a carboxylate monoanion at neutral pH. Using a Phos-tag derivative, Phos-tag acrylamide (**Figure 1A**), [2–4] commercially available from FUJIFILM Wako, Tokyo, Japan) in combination with Laemmli's sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [5], phosphate affinity electrophoresis (Phos-tag SDS-PAGE) has already been developed to detect shifts in the mobility of phosphoprotein species relative to their non-phospho counterparts (**Figure 1B**). To date, Phos-tag SDS-PAGE has been widely used to determine the phosphorylation state of many proteins [6–17]. The main advantages of Phos-tag SDS-PAGE are summarized in **Table 1**.

Casein is a type of dietary protein derived from cow's milk (CM). It is also the main ingredient in most dairy products and is the cause of many CM allergies [18–22].

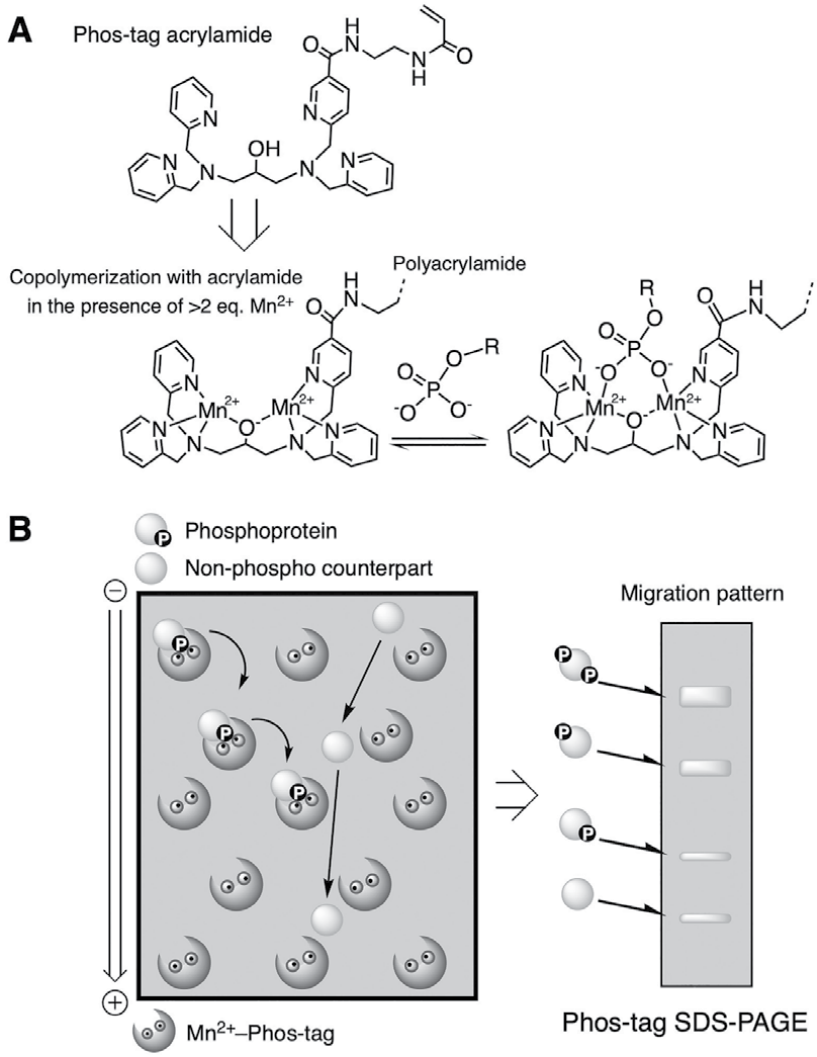

Figure 1.
Structure of Phos-tag acrylamide and scheme for the reversible capture of a phosphomonoester dianion ($R\text{-}OPO_3^{2-}$) using a polyacrylamide-bound Phos-tag (A). Schematic representation of the principle of Phos-tag SDS-PAGE (B).

The procedure is almost identical to conventional Laemmli's SDS-PAGE.
Downstream procedures such as gel staining, immunoblotting or MS analysis can be applied.
No radioactive or chemical labeling is required for protein kinase and protein phosphatase assays.
The specificity of phosphate binding is independent of the nature of the phosphorylated amino acid.
The time course of the quantitative ratio of phosphoproteins to non-phospho counterparts can be determined.
Multiple phosphorylated forms of a given protein can be detected as multiple migration bands, depending on the phosphorylation status.
Different phosphorylated forms of a single protein with the same number of phosphate groups can be separated.

Table 1.
Key advantages of Phos-tag SDS-PAGE.

When ingested, casein peptides can trigger an immune system response that can lead to a variety of symptoms. Casein allergy is most common in children and the symptoms can be easily mistaken for other common illnesses. Diagnosis at the first sign of symptoms is therefore important. It is known that α-casein and β-casein, which are members of the casein phosphoproteins, contain a highly homologous amino acid sequence "SSSEE" consisting of three serine residues and two glutamic acid residues as the core motif of the casein phosphopeptide [18, 19]. A very small amount of the tryptic digest containing the core motif has been reported to induce allergic symptoms in patients with CM allergy [20]. In addition, it has been suggested that the phosphate groups on the serine residue in the core motif are key to the binding of IgE antibodies in allergic patients [21]. Despite these reports, IgE-binding epitopes in the core motif have rarely been identified in epitope mapping studies of casein phosphoproteins. Our phosphate affinity electrophoresis technique could be used to map IgE-binding epitopes and potentially aid in this diagnosis.

This chapter describes the resolving power of Phos-tag SDS-PAGE for highly sensitive separation and detection of multiple phosphospecies of milk phosphoproteins, α-casein and β-casein, as typical protein samples involved in CM allergy, and discusses the potential utility of Phos-tag SDS-PAGE for mapping the critical IgE binding epitopes affected by phosphorylation.

2. Separation and detection of phosphoproteins by Phos-tag SDS-PAGE

Laemmli's SDS-PAGE (normal SDS-PAGE) [5] was performed using an Atto's PAGE apparatus AE6500 (Atto, Tokyo, Japan). For Phos-tag SDS-PAGE, 50 μM Phos-tag acrylamide and 2 equivalents of $MnCl_2$ (100 μM) were added to the resolving gel as previously described [2–4]. Other electrophoretic procedures were nearly identical to the conventional Laemmli method. Protein samples (0.25 mg) were treated with or without 20 U alkaline phosphatase (ALP) at 37°C for 5 h as previously reported [2, 3]. To stop ALP reactions, samples were dissolved in sample loading dye solution for SDS-PAGE and heated at 95°C for 5 min.

Figure 2 shows the effect of the Mn^{2+}–Phos-tag on the mobilities of the milk phosphoproteins, α-casein and β-casein, and an egg white phosphoprotein, ovalbumin, in electrophoretic gels. In normal SDS-PAGE, the mobilities of the α-casein, β-casein and ovalbumin phosphoproteins (ALP, −) and their dephospho counterparts (ALP, +) were nearly equal and therefore did not separate efficiently. Meanwhile, exaggerated mobility shifts of the phosphoproteins relative to their dephospho counterparts were confirmed in Phos-tag SDS-PAGE. Since bovine serum albumin (BSA) is a non-phosphoprotein and is used as a control sample, there is no difference in the degree of migration (R_f) between BSA treated with (+) or without (−) ALP in the gel. These results show that the migration degrees of phosphoproteins are smaller than those of their dephospho counterparts in the Mn^{2+}–Phos-tag co-polymerized resolving gel.

3. Quantitative determination of the dephosphorylation reaction of phosphoproteins by Phos-tag SDS-PAGE

We quantitatively monitored the enzymatic removal of phosphate groups from phosphoproteins using Mn^{2+}–Phos-tag SDS-PAGE (**Figure 3**). Time-dependent

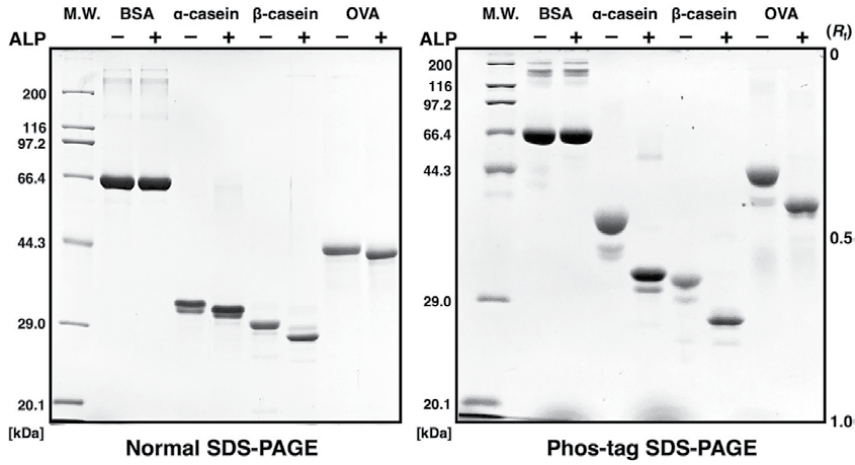

Figure 2.
SDS-PAGE followed by Coomassie Brilliant Blue staining of phosphoproteins (–) and their dephospho counterparts treated with ALP (+) in the absence and presence of polyacrylamide-bound Mn^{2+}–Phos-tag (50 μM). The R_f value of 1.0 is defined as the position of the bromophenol blue dye. M.W.: molecular weight markers (200: myosin, 116: β-galactosidase, 97.2: phosphorylase B, 66.4: bovine serum albumin, 44.3: ovalbumin, 29.0: carbonic anhydrase, 20.1: trypsin inhibitor), BSA: bovine serum albumin, OVA: ovalbumin.

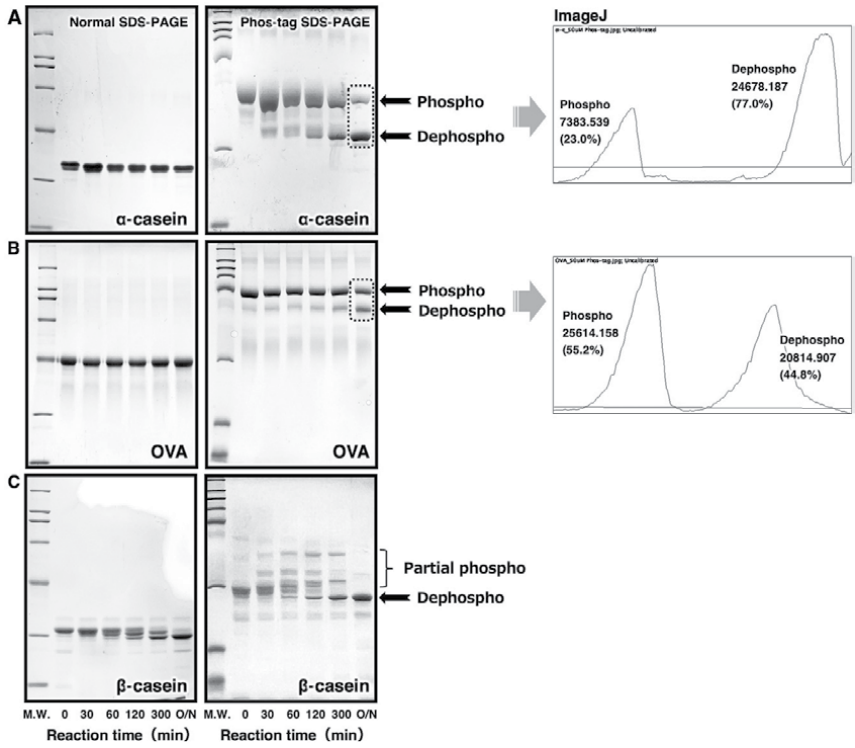

Figure 3.
Typical images of the time course of the dephosphorylation reaction of α-casein (A), ovalbumin (OVA, B), and β-casein (C) by normal SDS-PAGE and Phos-tag SDS-PAGE (50 μM Mn^{2+}–Phos-tag) followed by Coomassie Brilliant Blue staining. Results of densitometric analysis of overnight (O/N) samples of α-casein and ovalbumin are shown. M.W.: the same molecular weight markers as used in experiments of ***Figure 2****.*

dephosphorylation reactions of phosphoproteins (0.75 mg) were treated with 0.60 U ALP at 37°C as previously reported [2, 3]. Electrophoresis was performed as described above. Normal SDS-PAGE showed that the degree of migration and the amount of each sample in the phosphatase assays were almost equal. On the other hand, Phos-tag SDS-PAGE of α-casein and ovalbumin, shown in **Figure 3A** and **B**, respectively, showed that two migration bands were clearly visualized and the lower bands corresponding to their dephospho counterparts increased in a time-dependent manner. Densitometric analyses using ImageJ, a public domain image processing program available as a downloadable application (https://imagej.net/ij/index.html), showed that the quantitative ratio of phosphoproteins or dephospho counterparts to total proteins can be determined, and the quantitative ratios of the dephosphorylated forms produced by ALP to total proteins could be estimated to be 77.0% for α-casein and 44.8% for ovalbumin (see ImageJ densitometric data).

In the time course of the dephosphorylation reaction of β-casein (**Figure 3C**), several bands with different phosphorylation states were visualized by Phos-tag SDS-PAGE. Since there are different susceptibilities to ALP activity depending on the five phosphorylated serine residues within the β-casein molecule, the dephosphorylation reaction might be proceeded in a stepwise manner. They were then completely dephosphorylated overnight (O/N).

These results demonstrate that Phos-tag SDS-PAGE allows the simultaneous detection of phosphoproteins and their corresponding dephosphoproteins and the quantitative determination of the ratio of phosphoproteins/dephosphoproteins to total proteins in a polyacrylamide gel. In the next section, we describe in more detail the separation analysis of the β-casein phosphospecies using the phosphate affinity technique.

4. Two-dimensional phosphate affinity PAGE for the analysis of β-casein phosphospecies

The time-course dephosphorylation reaction of pentaphosphorylated β-casein was treated with 1.2 mU ALP at 37°C for 10–720 min, as previously reported [16]. To stop the reaction at the desired times, samples were dissolved in sample loading dye solution for urea-PAGE and heated at 95°C for 5 min. For urea-PAGE, 4.0 M urea was added to both the stacking gel and the resolving gel. Electrophoresis was performed on the same Atto AE-6500 instrument as described above. On urea-PAGE, the β-casein sample appeared as six bands with different numbers of phosphate groups and was completely dephosphorylated by 720 min (**Figure 4A**). The number of phosphate groups is indicated as nP (n: 0–5) on the right side of the gel. In normal SDS-PAGE, the degree of migration of each sample was almost the same (R_f value = 0.7, **Figure 4B**). In contrast, multiple bands were clearly observed in Mn^{2+}–Phos-tag SDS-PAGE (**Figure 4C**). The number of bands was greater than that observed in urea-PAGE. Interestingly, several bands with smaller R_f values than the fully phosphorylated form of β-casein were observed between 10 and 300 min, and the smallest band (upper arrowhead at an R_f value of 0.3) was still present at 300 min.

We then performed 2-DE coupling urea-PAGE and Mn^{2+}–Phos-tag SDS-PAGE to investigate the relationship between phosphate stoichiometry and the degree of migration of β-casein phosphospecies in Mn^{2+}–Phos-tag SDS-PAGE (**Figure 4D**). For 2-DE, as previously described [16], the lane was excised from the first-dimensional urea-PAGE gel and immobilized with agarose on the second-dimensional

Phos-tag SDS-PAGE gel. The 2-DE image allowed easy assignment of the correspondence between the bands separated in the urea-PAGE gel and those separated in the Phos-tag gel. In addition, the number of spots on the 2-D gel was greater than the number of bands on the 1-D urea gel or the 1-D Phos-tag gel. The result of 2-DE showed that the combination of urea-PAGE and Mn^{2+}–Phos-tag SDS-PAGE provided a better separation of the β-casein phosphospecies than either 1-D method.

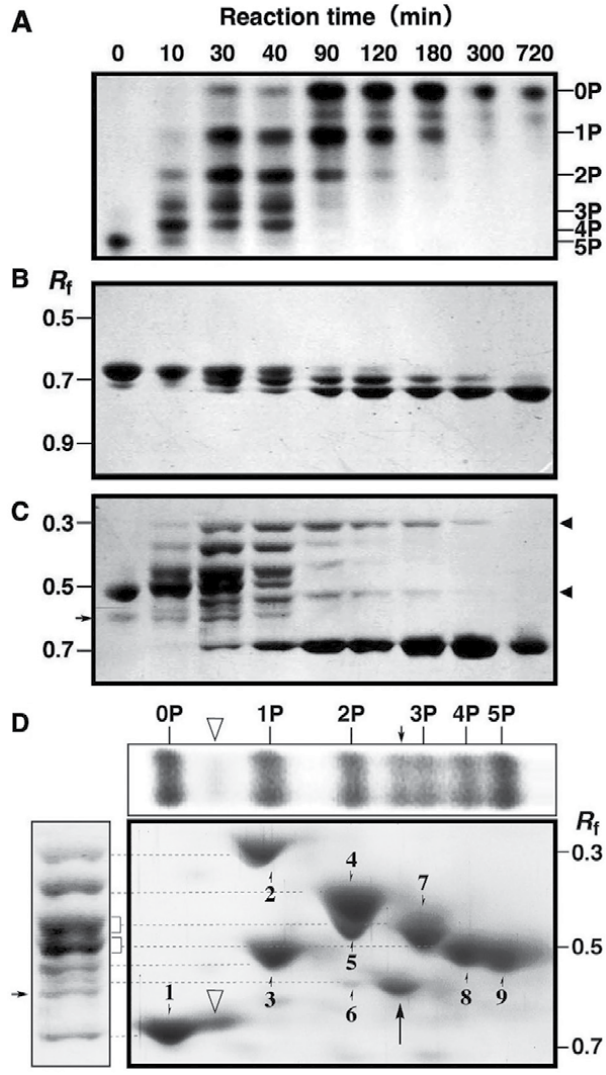

Figure 4.
Typical images of the time course of the dephosphorylation reaction of β-casein by urea-PAGE (A), normal SDS-PAGE (B) and Mn^{2+}–Phos-tag SDS-PAGE (100 μM Mn^{2+}–Phos-tag) (C) followed by SYPRO Ruby gel staining, and typical 2-D separation of the β-casein phosphospecies by coupling urea-PAGE and Mn^{2+}–Phos-tag SDS-PAGE (100 μM Mn^{2+}–Phos-tag) (D) followed by SYPRO Ruby gel staining. The migration spots on the 2-D gel are numbered from 1 to 9. The top panel corresponds to the 1-D urea-PAGE using a mixed sample of the fully phosphorylated, fully dephosphorylated and partially phosphorylated forms of β-casein. The left panel corresponds to the 30 min lane of ***Figure 4C****. The R_f value of 1.0 is defined as the position of the bromophenol blue dye. Reproduced with permission from Ref. [16] © (2009) Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim.*

5. Downstream application of LC-MS/MS to determine the phosphorylation site

To determine the phosphorylation site in the β-casein phosphospecies with the same phosphate stoichiometry, spots 2 and 3 in **Figure 4D** were applied to a downstream application of LC-MS/MS analysis. After in-gel tryptic digestion as described previously [16], the digests were analyzed using a nano LC-MS/MS system coupled to a Chorus220 nano HPLC pump and an HTC-PAL autosampler (CTC Analytics AG, Zwingen, Switzerland) and a hybrid triple quadrupole/linear ion trap tandem mass spectrometer, 4000QTRAP (Applied Biosystems, Foster City, CA, USA). As a result, monophosphopeptides of FQpSEEQQQTEDELQDK (amino acid sequence = 48–63 residues) and RELEELNVPGEIVESLSSpSEESITR (amino acid sequence = 16–40 residues) were identified from spots 2 and 3, respectively. Two phosphorylation sites of Ser-50 and Ser-34 residues were identified separately from the different spots. No MS signal corresponding to non-phosphopeptides or other phosphopeptides in the same digests was detected. Spots 2 and 3 were therefore assigned to monophosphorylated β-casein species with a different phosphorylation site. This showed that Mn^{2+}–Phos-tag SDS-PAGE allowed protein phosphospecies with the same phosphate stoichiometry to separate as distinct bands due to the difference in phosphorylation site. In addition, the downstream application of MS analysis revealed that the Ser-50 and Ser-34 residues are phosphorylation sites that are not easily affected by ALP activity, as spots 2 and 3 correspond to the smallest migration band (an R_f value of 0.3) and the fifth band (an R_f value of about 0.5), respectively, from the top of the 1-D Mn^{2+}–Phos-tag SDS-PAGE, still observed at reaction times of 120–300 min (see upper and lower arrowheads in **Figure 4C**).

6. Conclusions

This chapter first outlines the basic principle of phosphate affinity electrophoresis using Phos-tag acrylamide and describes the resolving power of Phos-tag SDS-PAGE for the highly sensitive separation and detection of multiple phosphospecies of milk phosphoproteins as typical protein examples.

In the milk phosphoproteins, α-casein and β-casein, the presence of a highly homologous amino acid sequence consisting of three serine and two glutamic acid residues, "SSSEE", is known as the core motif of the casein phosphopeptide [18, 19]. It has been reported that the tryptic digestion product containing the core motif, which is the identical peptide identified from the in-gel tryptic digestion of spot 3 in our 2D gel (see **Figure 4D**), induces allergic symptoms in patients with CM allergy, albeit at very low levels [20]. Furthermore, it has been suggested that the phosphate groups on the serine residue in the core motif play an important role in the binding of IgE antibodies in allergic patients [21]. From the result of our study, we found that one of the phosphorylated serine residues within the motif (phosphorylated Ser-34) is not easily affected by the dephosphorylation reaction with ALP activity. This may be related to the effect of the phosphate groups in β-casein on IgE binding capacity as described previously [22]. By using sera from patients with CM allergy, our phosphate affinity electrophoresis technique may help to map the IgE binding epitopes.

The Phos-tag SDS-PAGE system uses the same equipment and reagents as conventional Laemmli SDS-PAGE and can also be adapted for downstream procedures such as immunoblotting using specific antibodies against specific targets. As a result,

many researchers from basic life sciences to clinical medicine have used this system to report valuable insights into protein phosphorylation modifications [6, 17]. We expect that our Phos-tag-based method will contribute to the diagnosis of many diseases, including CM allergy, which is associated with protein phosphorylation, as well as to the screening of phosphorylation regulators for drug discovery.

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Author details

Eiji Kinoshita[1*] and Emiko Kinoshita-Kikuta[2]

1 Faculty of Human Sciences, Department of Human Nutrition, Hiroshima Bunkyo University, Kabehigashi, Asakita-ku, Hiroshima, Japan

2 Department of Functional Molecular Science, Graduate School of Biomedical and Health Sciences, Hiroshima University, Kasumi, Minami-Ku, Hiroshima, Japan

*Address all correspondence to: kinoshitae@h-bunkyo.ac.jp

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Milk proteins and their interactions with other components, such as lactose, are very important, offering vast potential across various industries. They are now integral in the development of pharmaceuticals, cosmetics, and baked goods, showcasing their versatility and importance in modern industry. This book explores the latest advancements in milk protein research, focusing on innovative extraction techniques and methods for preserving and modifying protein functionality. Key processes such as heat treatment, enzyme treatments, and hydrolysis are thoroughly explored to optimize the functional attributes of milk proteins for diverse applications. Driven by environmental and sustainability concerns, research has increasingly prioritized the development of greener dairy processing practices. These sustainable approaches aim to reduce waste and improve efficiency, contributing to the broader goal of environmentally friendly milk protein ingredients and lactose. This book provides valuable insights into the complex interactions between milk proteins and other milk components, such as lactose, offering a comprehensive guide to harnessing these interactions for innovative applications and sustainable practices.

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