# Peptidases used in dairy technology: Current knowledge and relevant applications

Peptidases utilizadas na tecnologia de laticínios: Conhecimento atual e aplicações relevantes

Peptidasas utilizadas en tecnología láctea: Conocimiento actual y aplicaciones relevantes

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**Abstract**
The dairy sector is one of the most important industrial segments in peptidase applications These enzymes can hydrolyze milk proteins into medium/short peptides and amino acids, as well as modulate their nutritional and functional properties, which comprise sensory changes (e.g., texture and flavor), digestibility and solubility improved, as well as the release of bioactive compounds. Therefore, they have been applied to develop different dairy products, such as cheese and a wide range of products deriving from caseins and whey proteins. However, it is important to understand the structure of milk proteins at the time to select the best peptidase to achieve the desired hydrolyzed products. In addition, peptidases have different specificities, such as catalytic sites and optimal pH, which must be taken into account before their application in the dairy matrix. The present review aims to address important aspects associated with peptidase features and their current biotechnological applications in the dairy industry.
**Keywords**: Exogenous enzyme; Dairy processing; Milk protein hydrolysates; Enzymatic hydrolysis; Proteolysis.

**Resumo**
O setor de laticínios é um dos segmentos industriais mais importantes em aplicações de peptidases. Essas enzimas são capazes de hidrolisar proteínas do leite em peptídeos médios/curtos e aminoácidos, bem como modular suas propriedades nutricionais e funcionais, as quais compreendem alterações sensoriais (por exemplo, textura e sabor), melhorias nas características de digestibilidade e solubilidade, além da liberação de compostos bioativos. Portanto, as peptidades têm sido aplicadas no desenvolvimento de diversos produtos lácteos, como queijos e outros produtos derivados das caseínas e proteínas de soro de leite. No entanto, é importante conhecer a estrutura das proteínas do leite a fim de selecionar a melhor peptidase visando a obteção dos hidrolisados desejados. Além disso, as peptidases possuem diferentes especificidades, como sítios catalíticos e pH ótimo de atuação, que devem ser levados em consideração antes de sua aplicação na matriz láctea. A presente revisão visa abordar aspectos importantes associados às características das peptidases e suas aplicações biotecnológicas atuais na indústria de laticínios.
**Palavras-chave**: Enzima exogen; Processamento de lácteos; Hidrolisados das proteínas do leite; Hidrólise enzimática; Proteólise.

**Resumen**
El sector lácteo es uno de los segmentos industriales más importantes en aplicaciones de peptidasas. Estas enzimas son capaces de hidrolizar las proteínas de la leche en péptidos y aminoácidos medianos/cortos, así como modular sus propiedades nutricionales y funcionales, que incluyen cambios sensoriales (por ejemplo, textura y sabor), mejoras en las características de digestibilidad y solubilidad, además de liberación de compuestos bioactivos. Por lo tanto, los péptidos se han aplicado en el desarrollo de diversos productos lácteos, como quesos y otros productos derivados de la caseína y

las proteínas del suero. Sin embargo, es importante conocer la estructura de las proteínas de la leche para seleccionar la mejor peptidasa para obtener los hidrolizados deseados. Además, las peptidasas tienen diferentes especificidades, como sitios catalíticos y pH óptimo de acción, que deben tenerse en cuenta antes de su aplicación en la matriz láctea. La presente revisión tiene como objetivo abordar aspectos importantes asociados con las características de la peptidasa y sus aplicaciones biotecnológicas actuales en la industria láctea.

**Palabras clave:** Enzima exógena; Procesamiento de lácteos; Hidrolizados de proteína de leche; Hidrólisis enzimática; Proteólisis.

## 1. Introduction

Proteases, proteinases, or peptidases form a group of hydrolytic enzymes capable of cleaving peptide bonds in proteins and peptides (Barret & McDonald, 1986). There are almost no meaningful differences among these terminologies, although using a single term could guarantee access to all current data and consequently provide correct scientific information. Thus, some authors see peptidase as the most suitable term, which is subdivided into exopeptidase and endopeptidase (Barret & McDonald, 1986; Barrett, 1999; Barrett, 2000). In addition, this terminology is recommended by the International Union of Biochemistry and Molecular Biology (IUBMB) (Barrett, 1999; Barrett, 2000; IUBMB, 1992).

Peptide bond hydrolases prevail among enzymes applied in the industrial segment (Gurumallesh et al., 2019; Gurung, 2013; Mazorra-Manzano et al., 2020) since they account for more than 50% of the global enzyme market and their growth rate is expected to reach 4.9% at compound annual growth rate (CAGR) by 2027 (Research and Markets Report, 2021). The high industrial interest in using peptidases is partly explained by the fact that these enzymes are an alternative to replace chemical treatments; therefore, they can contribute to mitigate environmental impacts (Tavano, 2015). Furthermore, peptidases' action is quite specific, since they can help preserving other substrate components by interfering in raw material the least possible.

These enzymes play an essential role in different industrial sectors; they can be applied in the dairy industry to produce different cheese types or milk protein hydrolysates (Table 1).

**Table 1** – Main commercial peptidases applied in dairy industries.

| Featured Suppliers | Trade name | Dairy application |
|---|---|---|
| AB Enzymes (Germany) | Corolase® 7089 | Milk protein hydrolysate |
| | Corolase® 8000 | Milk protein hydrolysate |
| | Corolase® 2TS | Milk protein hydrolysate |
| Advanced Enzyme (USA) | Corolase® 2TS | Milk protein hydrolysate |
| | SEBCheese Pro | Microbial coagulant |
| | FlavourSEB NP | Dairy processing peptidase |
| Biocatalyst Limited (United Kingdom) | Promod™ 517MDP | Milk protein hydrolysate |
| | Promod™ 903MDP | Manufacture of Enzyme-modified cheese |
| | Promod™ 845MDP | Manufacture of Enzyme-modified cheese |
| | Promod™ 782MDP | Whey protein hydrolysate |
| | Promod™ 523MDP | Whey protein hydrolysate |
| | Promod™ 439L | Whey protein hydrolysate |
| | Promod™ 215MDP | Manufacture of Enzyme-modified cheese |
| | Flavorpro™750MDP | Whey protein hydrolysate |
| | Flavorpro™766MDP | Debittering |
| | Flavorpro™937MDP | Debittering |
| | Flavorpro™ Umami | Manufacture of Enzyme-modified cheese |
| Chr.Hansen (Denmark) | Microlant® | Cheese manufacture (Microbial source coagulant) |
| | Chy-max® | Cheese manufacture (Fermented chymosin) |
| | Far-m® | Cheese manufacture (Coagulant – animal source) |
| | Naturen® | Cheese manufacture (Coagulant – animal source) |

| DSM (Netherlands) | EndoPro® | Debittering and milk protein hydrolysate |
|---|---|---|
| | Maxiren® | Cheese manufacture (Recombinant chymosin) |
| | Fromase® | Cheese manufacture (Coagulant - microbial source) |
| | Kalase® | Cheese manufacture (Coagulant – animal source) |
| | Milase® | Cheese manufacture (Coagulant - microbial source) |
| | Accelerzyme® | Accelerating cheese ripening |
| IFF Nutrition & Biosciences (USA) | Carlina™ | Cheese manufacture (Coagulant - animal source) |
| | Debitrase® | Whey processing (Reduces bitterness) |
| | Marzyme® | Cheese manufacture (Coagulant - microbial source) |
| Enmex SA DE CV (Mexico) | Laczyme ® | Cheese manufacture (Coagulant - microbial source) |
| Prozyn (Brazil) | Protamex® | Dairy protein hydrolysate |
| | Quimozyn ® | Cheese manufacture (Coagulant – fermented chymosin, |

Source: Authors.

The use of exogenous peptidases, such as chymosin, to convert unprocessed milk into cheese is very well established in the literature and industry (Garcia et al., 2017). However, the discovery of new peptidases, and their action mechanism on milk proteins, enabled the dairy industry to implement oriented modifications on proteins' structure and promote positive changes in the nutritional, physicochemical, and techno-functional properties of milk proteins (De Castro et al., 2015).

Thus, the use of peptidases is prospering in the dairy sector, however, it is important complying with safety guidelines and regulations, as well as take into consideration the variability in peptidases' specificities, at the time to introduce them in the dairy market. Furthermore, aspects such as the protein source to be hydrolyzed and the predicted hydrolysates must be taken into account (Tavano, 2013). The current review aimed to present the main peptidase applications in the dairy industry and to highlight some crucial aspects to be taken into consideration at the time to determine the peptidase of choice for dairy technology use.

## 2. Methodology

This review was based on the research of scientific articles from different indexing bases, regarding the main characteristics of peptidases and their biotechnological applications in dairy products. The papers adopted for the construction and discussion of this review include the most relevant and current works on peptidase in dairy technology.

## 3. Results and Discussion

### 3.1 Peptidases' features

Peptidases catalyze the cleavage of peptide bonds capable of linking amino acids to the polypeptide chain of a given peptide or protein structure (Figure 1). Polypeptides, short peptides, and isolated amino acids can be released as hydrolysis products (Güler et al., 2016).

**Figure 1** - Example of a hydrolysis reaction of a carbon-nitrogen bond.
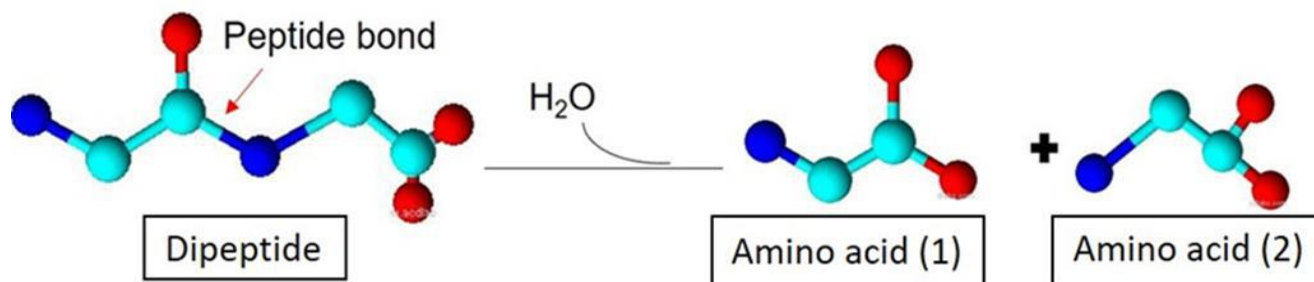


Source: Adapted from Mótyán et al. (2013).

The international enzyme nomenclature and classification system (Enzyme Commission Number - EC number) classifies peptidases as hydrolases (group 3) belonging to subgroup 4. This classification indicates that peptidases act in peptide bonds, whereas the last two digits of the EC number refer to their enzymatic catalysis mechanism (EC 3.4.-.-) (Ehrmann & Clausen 2004; Mótyán et al., 2013). EC classification does not take into account structural peptidase groups reflecting evolutionary relationships. A new form of classifying this class of enzymes, based on their essential structural features, was designed around 1992 and was published as MEROPS database (http://www.merops.co.uk) in 1996. Based on this classification, each peptidase family is named with a letter referring to its catalytic type, namely: aspartic (A) peptidase, cysteine (C) peptidase, glutamic (G) peptidase, metallo (M) peptidase, asparagine (N) peptide lyases, mixed (P) peptidase, serine (S) peptidases, threonine (T) peptidase and unknown (U) catalytic-type peptidases. In addition to the aforementioned classification systems, peptidases can be categorized as alkaline (pH ranging from 8.0 to 13.0), neutral (pH ranging from 6.0 to 8.0), or acidic (pH ranging from 2.0 to 6.0) based on their optimal catalytic pH (Vranova et al., 2013). Peptidases are subdivided into exopeptidases or endopeptidases, depending on aspects such as reaction type and substrate interaction (Figure 2).

**Figure 2** - Classification of peptidases based on their site of action.
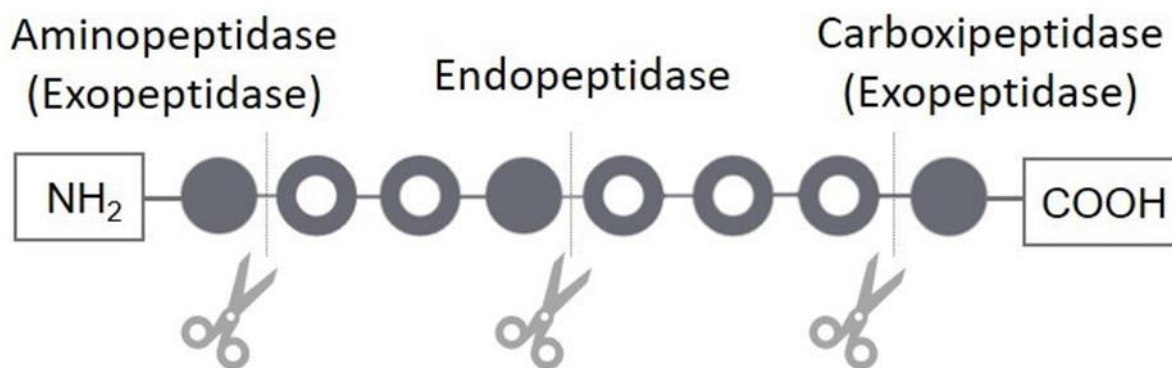


Source: Authors.

Exopeptidase (EC 3.4.11-19) performs hydrolysis near nitrogen (aminopeptidase) or carbon (carboxypeptidase) terminals embodied in the substrate to produce mono amino acid, dipeptide or tripeptide residues (Mótyán et al., 2013; Tao et al., 2011). Carboxypeptidases can be subdivided into serine-type carboxypeptidases, metallocarboxypeptidases, cysteine-type carboxypeptidases and dipeptidases; whereas aminopeptidases comprise dipeptidyl- and tripeptidyl-peptidases (Barrett, 1999;

Gurumallesh et al., 2019). Some enzymes present both features - i.e., carboxypeptidase and aminopeptidase catalytic activity - because their structure presents negative and positive forms, wherein the negative charge binds to the N- terminus, whereas the positive charge binds to the negatively charged C-terminus of a given substrate (Tavano, 2013).

On the other hand, endopeptidases (EC 3.4.21-24 together with 3.4.99) act in the cleavage of non-terminal amino acids (Sawant & Nagendran, 2014). It means that the peptide substrate runs through the whole extent of the active site of the peptidase structure and is cleaved somewhere at its midpoint (McDonald, 1985). Peptidases can be clustered into six classes based on the chemical type of the group that is primarily in charge of the catalytic activity, namely: cysteine, serine, glutamic acid, aspartic acid, metallo or threonine peptidases (Table 2) (Gurumallesh et al., 2019). All these peptidases' features - i.e., reaction type, substrate interaction, catalytic site, optimal pH and diversity specificities - will determine their biotechnological use.

**Table 2**- Endopeptidases specifications.

| Peptidase | Amino acid residues in the active site | Ec no | Inhibitors examples | pH optimum | Examples |
|---|---|---|---|---|---|
| Aspartic | Aspartate and cysteine | 3.4.23 | Pepstatin and in the presence of copper ions | 3-4 | Pepsin, chymosin and microbial aspartic peptidases |
| Cysteine | Aspartate, cysteine, and histidine | 3.4.22 | Sulfhydryl reagents, eg:4-hydroxy mercury benzoic acid | 2–3 | Papain and bromelain |
| Glutamic acid | Glutamic acid and glutamine | 3.4.19 | Pepstatin | 2 – 3.5 | Fungal peptidases |
| Metallopeptidases | Histidine, glutamine, aspartate, and cysteine | 3.4.24 | Chelating agents (eg: EDTA) | 5–8 | Collagenase, elastase, thermolysin |
| Serine | Serine, histidine and aspartate | 3.4.21 | EDTA, trypsin inhibitor, phosphate buffer, phenols. | 7- 11 | Trypsin and chymotrypsin |
| Threonine | Threonine | 3.4.25 | Dipeptide boronic acid, Epoxyketones | 6.5 – 7.5 | Acyltransferases and proteasome |

Source: Adapted from: Gurumallesh et al. (2019).

Endopeptidase or exopeptidase application in industrial processing depends on the intended hydrolysates; in some cases, the combined use of endo and exopeptidases can provide the best result. Protein hydrolysis used to manufacture whey protein hydrolysates (free amino acids and short peptides) is triggered by endopeptidases in order to increase the number of terminal peptide sites; then, it is completed by exopeptidases (Clemente, 2000; Cui et al., 2022).

Moreover, it is worth emphasizing differences in peptidase specificity. Some peptidases can hydrolyze the structure of peptides and proteins at distinct peptide bonds regions, whereas others are much more specific since they only attack a single amino acid sequence. Thus, if a specific peptide is the target of a milk protein hydrolysate, such as bioactive peptides, it is necessary selecting the most suitable peptidase presenting the proper narrow specificity (Tavano et al., 2018).

**3.2 Requirements for peptidase application in the dairy industry**

Peptidases derive from three main sources: plants, animals, and microbes. However, microorganisms are preferably used to meet significant industrial demands because they can achieve high proteolytic enzyme yield by spending lesser time, space and investments than plant or animal sources (Dhillon et al., 2017). Approximately 50 microorganisms are overall acknowledged by FDA as safe (GRAS) for enzyme production purposes. Among them, one finds bacteria and fungi, which are mainly represented by genera Bacillus and Aspergillus, respectively (Singh et al., 2016).

New commercial peptidases, regardless of their source, must fulfill safety recommendations by following guidelines provided by the Food and Agriculture Organization of the United Nations (FAO), the World Health Organization (WHO), the Food Chemicals Codex (FCC), and by the Association of Manufacturers and Formulators of Enzyme Products (AMFEP) in Europe; by the Enzyme Technical Association (ETA) in the US (Gurung et al., 2013), and by the National Health Surveillance Agency (ANVISA) in Brazil. These guidelines aim at ensuring the safety of enzyme preparations for consumption purposes. In addition, they may include specifications concerning enzymes' purity and activity (Spök, 2006). For example, it is necessary investigating the enzyme source, since the microorganism strain to be used must be of the nonpathogenic type. Moreover, diluents and other ingredients used in enzyme production processes must be acceptable for dietary purposes (FAO, 2021).

Based on these requirements, the process to select a given peptidase for a dairy application depends on several factors (Figure 3). Therefore, understanding the specificity of a given enzyme and classifying it are the starting points to select it (Tavano, 2015).

**Figure 3** – Aspects to determine a peptidase for a dairy application.
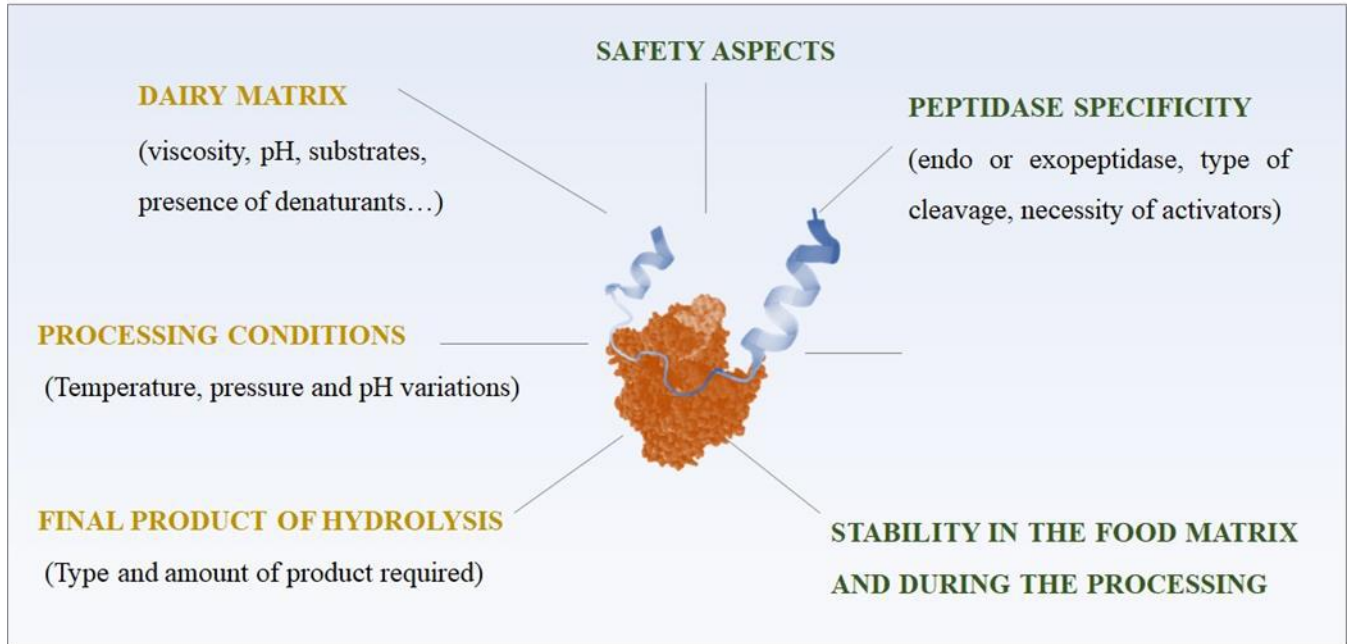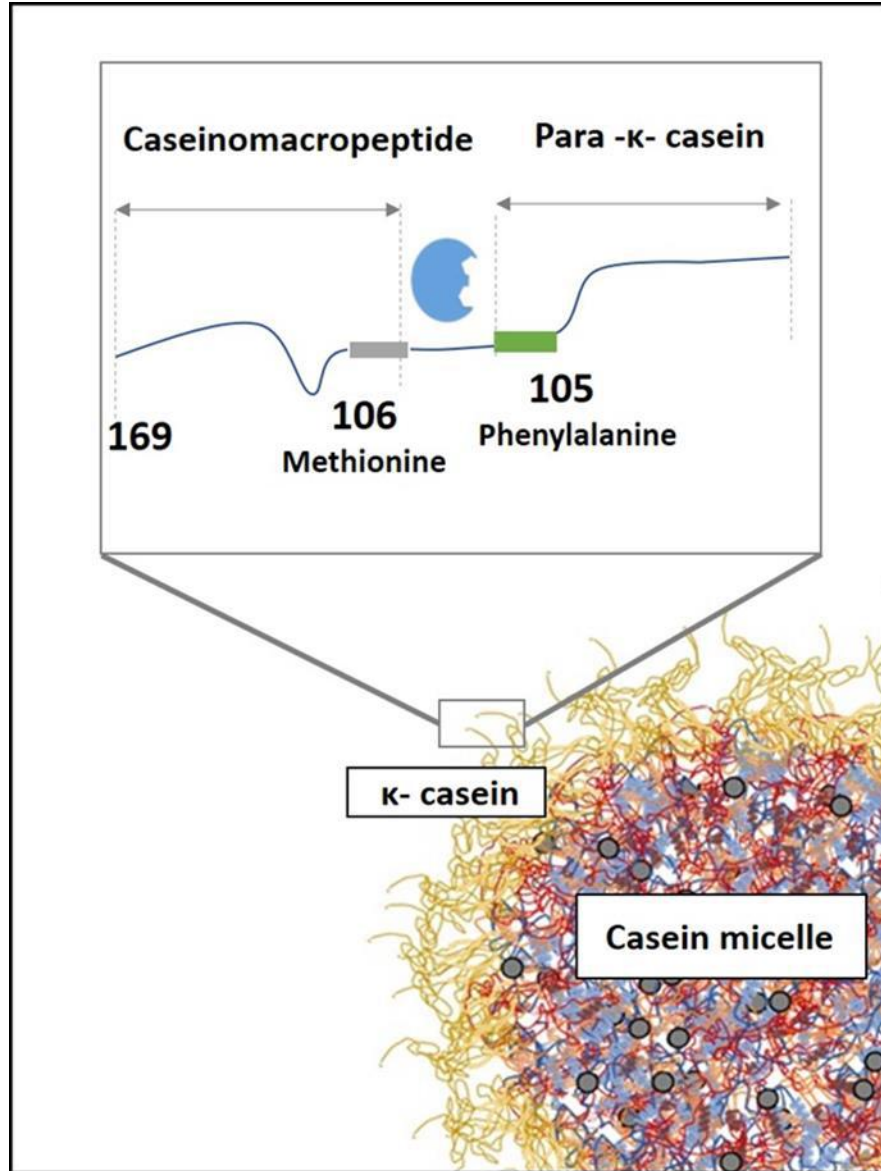


Source: Authors.

## 3.3 Main peptidase applications in the dairy industry

### 3.3.1 Cheese manufacturing

One of the main peptidase applications in dairy production lies on using rennet in cheese manufacturing processes (Abada, 2019). Rennet derives from the stomach of ruminants, such as calves and adult cattle (Horne & Lucey, 2017). Calf rennet comprises a complex blend of aspartic peptidases, mainly chymosin and pepsin (Garcia et al., 2017; Merheb-Dini et al., 2012). Chymosin has specific action in casein and presents remarkably high milk clotting activity with low proteolytic action (Garcia et al., 2017; Sanchez & Demain, 2017; Visser, 1993). This peptidase catalyzes the cleavage of the specif κ-casein region between amino acids 105 and 106 (Figure 4). This process leads to decreased repulsion forces (electrostatic and steric) between caseins, which, in their turn, lead to their destabilization and contribute to milk coagulation (Gomes et al., 2018; Nongonierma & FitzGerald, 2011; Tavano, 2013).

**Figure 4** - Chymosin's action mechanism.



Source: Authors.

Bovine pepsin is less substrate-specific; it hydrolyzes bonds with Phenylalanine, Tyrosine, Leucine or Valine residues and is more proteolytic than the corresponding chymosins (Agudelo et al., 2004; Fox & McSweeney, 1996). Excessive and nonspecific proteolysis may lead to yield loss and defects in cheese, such as weak gel structure and bitterness (Horne & Lucey, 2017). Thus, pepsin may lead to milk fat loss, since the resulting curd has a more open and loosen structure than that mostly formed with chymosin, which results in softer-body cheeses (Garcia et al., 2017). Therefore, chymosin and pepsin proportion in the rennet has a direct impact on cheese quality (Jacob et al., 2011).

The increasing demand observed in the cheese market, in association with expansive costs with animal rennet and with religious or dietary concerns about its consumption, have prompted the use of alternative peptidase sources with coagulant properties (Lemes et al., 2016; Zikiou & Zidoune, 2018). These milk-clotting enzymes must show properties similar to those of chymosin, such as specificity to hydrolyze κ-casein, and activity under the same temperature and pH conditions, without resulting

in bitter taste (Jacob et al., 2011). The major rennet substitutes meeting these requirements comprise microbial, recombinant and plant-based peptidases (Shah et al., 2014). Although recombinant chymosins were banned from several countries (Vallejo et al., 2012), they present 100% chymosin activity (Kumar et al., 2010) in comparison to conventional rennet, which presents approximately 20% pepsin activity.

Several studies reported calf rennet replacement, focused on founded milk-clotting enzymes deriving from microorganisms such as bacteria (Ahmed et al. 2016; Cavalcanti et al., 2004; Guleria et al. 2016; Lemes et al. 2016; Meng et al. 2018; Narwal et al. 2016; Shieh et al. 2009; Wehaidy et al. 2018; Wehaidy et al. 2020) and fungi (Hashem, 2000; Shamtsyan et al., 2014); or from plant sources such as fruits (Gagaoua et al., 2017; Grozdanovic et al., 2013; Mazorra-Manzano et al., 2013; Salehi et al., 2017,), seeds (Ahmed et al., 2009; Ahmed et al., 2016), flowers (Cavalli et al. 2013),  roots (Gagaoua et al. 2015; Gagaoua et al. 2016) and latex (Afsharnezhad et al. 2018; Kumari et al. 2012;). Most recently, Yang et al. (2022) have identified a likely new milk-clotting peptidase deriving from an insect.

Coagulants deriving from plant extracts have been added to milk for cheesemaking purposes since ancient times (Shah et al., 2014). The great advantage of cheeses prepared with plant coagulants lies on the fact that they are suitable for vegetarians or consumers with religious restrictions (Dupas et al., 2020). However, plant coagulants may present high proteolytic nature, and it can lead to lower cheese yield, as well as to sensorial changes, such as bitter flavor and texture defects (Salehi et al., 2017; Shah et al., 2014;). Therefore, microbial coagulants stood out among the analyzed ones, since they enabled large-scale production without raising environmental concerns (Gurumallesh et al., 2019).

### 3.3.2 Cheese ripening

Proteolysis is one of the most important biochemical events taking place during cheese ripening since it accounts for changes in cheese texture and flavor caused by smaller peptides and free amino acids release (McSweeney, 200 0).

Most milk-clotting enzymes added to milk are lost in the whey, but some of them remain in the curd and account for primary proteolysis (Tavano, 2013). Secondary proteolysis takes place throughout the ripening process (Fox et al., 1996). As seen in Figure 5, proteolysis takes place during cheese ripening based on the following steps: 1) casein is hydrolyzed into large peptides, mainly by the action of the enzymatic coagulant and some indigenous enzymes found in milk; 2) these large peptides are hydrolyzed into small peptides by microbial peptidases deriving from starter and non-starter microorganisms; 3) small peptides are hydrolyzed into amino acids by microbial peptidases, which generate flavor and aroma compounds.

**Figure 5** - Proteolysis steps during cheese ripening.
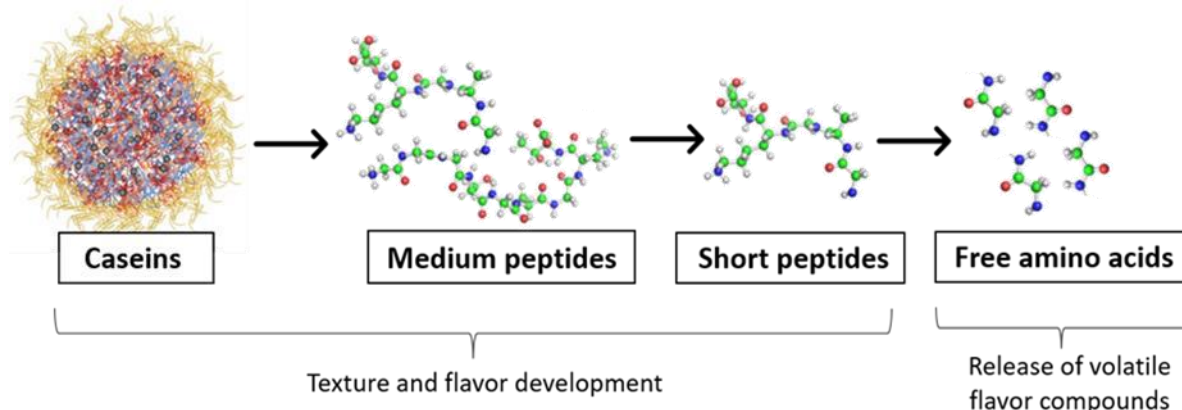


Source: Authors.

Proteolysis level during the ripening process depends on several factors, such as endogenous composition of milk, exogenous enzymes, enzymes found in coagulant, and enzymes produced by different microorganisms added in cheese milk (Fox et al., 1996). Adjunct cultures and non-starter lactic acid bacteria (NSLAB), mainly lactobacilli, are supplemented in cheesemaking processes due to their potential to release proteolytic enzymes capable of enhancing cheese flavor and texture, as well as of speeding up the ripening process (Soda & Awad, 2011). For example, blue cheeses have remarkably high-intensity proteolysis due to metallopeptidases and aspartic peptidases secreted by microorganism species *Penicillium roqueforti*, which is added to their manufacturing process. These peptidases degrade casein fractions until the entire casein micelle is gradually hydrolyzed into smaller peptides (Xia et al., 2020). Cagno et al. (2012) have investigated the use of NSLAB in a typical pasta filata cheese. Results of microbiological, biochemical and sensory analyses have shown that enzymes deriving from NSLAB were capable of speeding up the Cacioacavallo cheese ripening process, without changing the main features of this traditional cheese (Cagno et al., 2012).

Ripening conditions, such as temperature and humidity, must be carefully controlled to promote the desired development of microorganisms and enzymes' release (Soda & Awad, 2011). Ripening is a long and expensive process, which can take up to 2 years to be completed, depending on the cheese variety (Gripon et al., 1991). For example, Cheddar cheese ripening time ranges from 3 to 18 months (Kilcawley et al., 2012); each ripening month can increase its costs by up to 3% (Soda & Awad, 2011).

Using some specific peptidases to accelerate ripening time is one of the strategies adopted by commercial cheese-makers to reduce their costs (Nongonierma & FitzGerald, 2011; Tavano, 2013). As previously mentioned, these peptidases can come from adjunct cultures or may be directly added to cheesemilk or cheese curd in order to accelerate the ripening process and to avoid flavor defects (Khattab et al., 2019). However, peptidases added to cheesemilk are often lost in the whey; only a very small portion of them is retained in the curd (Azarnia et al., 2011; Soda & Awad, 2011). Therefore, it is preferable to use encapsulated enzymes rather than directly adding them to cheesemilk in order to avoid enzyme losses and poor distribution (Karel, 1990). Encapsulated enzymes are released over the ripening process, into the chesse curd, upon capsule breakdown (Karel, 1990). Azarnia et al. (2011) investigated the use of a recombinant aminopeptidase, in its encapsulated and free form, during Cheddar cheese ripening process. Results have shown that cheeses added with 2000 encapsulated enzyme units recorded significantly increased secondary proteolysis indices in comparison to those supplemented with free enzymes. Moreover, significant sensorial differences between them were observed; the highest mean scores recorded for texture, flavor and aroma were observed in cheeses supplemented with

encapsulated peptidase (Azarnia et al., 2011). However, it is important taking into consideration the safety and suitability of the encapsulation material for large-scale production.

### 3.3.3 Enzyme-modified cheeses (EMCs)

EMC development is another peptidase application in the dairy industry. These products are defined as concentrated cheese flavors, enzymatically manufactured (peptidases and lipases, or only peptidases) based on aged cheeses and other ingredients, such as casein blends, whey powder and skim milk powder (Moskowitz & Noelck 1987; Wilkinson & Kilcawley 2011). EMCs can speed up the cheese ripening process, however, they are mainly used to improve the flavor of different cheese and processed food types (Hannon et al., 2006). The demand for EMCs as flavor ingredients has increased due to their significant use by the food industry in low-fat and non-fat products, as well as to their pronounced flavor, which is up to thirty times stronger than that of natural cheese (Kilcawley et al., 2000).

EMC production is based on the cheese ripening process, which involves exogenous enzymes' addition to cheese curd under controlled conditions (Figure 6). Nowadays, many peptidases used for EMC manufacturing are available in the market, such as Promod™ 903MDP and Flavorpro™ Umami; both of them derive from *Aspergillus* sp (Table 1).

**Figure 6:** EMC manufacturing by the one-stage process.
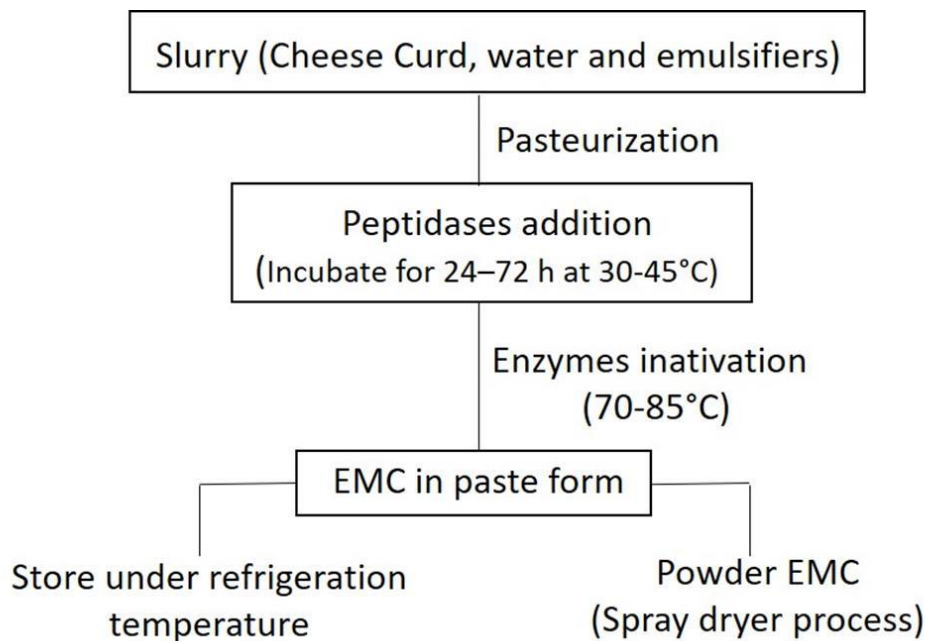


Source: Adapted from Wilkinson and Kilcawley (2011).

EMCs with different cheese flavors can be produced from the same material by changing their process conditions (Hannon et al., 2006). There are three main well-known EMC manufacturing approaches, namely: 1) one-stage process, which is based on simultaneous cheese curd proteolysis and lipolysis under controlled conditions; 2) a second process, according to which, proteolysis and lipolysis are individually carried out in different substrates (eg. butterfat/cream, for lipolysis; and cheese curd, for proteolysis); and 3) two-stage process, which uses a single stable substrate; it starts with proteolysis, which is followed by lipolysis (Bas et al., 2019; Kilcawley et al., 2006;). The one-stage process is the technique mostly used for commercial purposes among the

aforementioned processes (Bas et al., 2019).

EMCs come in both liquid and powder forms; however, powdered EMC is the preferred form due to its higher shelf-life and smaller volume to stock (Salum et al., 2022). Ali et al. (2022) used enzyme-modified cheese powder as ingredient in bread processing and showed that short- and medium-chain peptides and amino acids present in EMC had positive effects on bread aroma (Ali et al., 2022). High peptide and amino acid levels released during proteolysis account for the intense flavor observed in EMC (Hannon et al., 2006).

EMCs undergo more extensive protein hydrolysis than natural cheeses (Moskowitz & Noelck, 1987). Proteolysis extent and pattern can change depending on the used proteolytic enzyme type and on proteolysis levels during the EMC manufacturing process (Kilcawley et al., 2000). It is worth emphasizing that extensive and nonspecific proteolysis can release high bitter peptide levels. It may happen due to the accumulation of hydrophobic peptides deriving from β-casein, which are associated with increased bitterness (Park et al., 1995). According to Bas et al. (2019), hydrophobic peptides presenting 3–15 amino acids are the main source of bitterness in EMC. Thus, it is important selecting the proteolytic enzymes to be added to EMC, as well as understand their specific substrates, to avoid high bitter peptide levels in the product. Another strategy to avoid this issue lies in the use of specific peptidases capable of hydrolyzing bitter peptides into smaller peptides and amino acids (Park et al., 1995). Debittering peptidases available in the market (Table 1) often present exopeptidases which can hydrolyze bitter peptides generated by endopeptidase action (Biocatalysts, 2015).

### 3.3.4 Milk protein hydrolysates (MPH)

Milk protein concentrates/isolates are functional dairy powder ingredients produced by concentrating proteins in skim milk through ultrafiltration; this procedure is followed by evaporative concentration and spray drying process (Kelly, 2011; Oldfield & Singh 2005). The resulting product has high protein content, e.g.: milk protein concentrates (MPC; 85% protein in dry matter) and milk protein isolate (MPI; higher than 90% protein in dry matter) (Kelly, 2011).

These products can be hydrolyzed through peptidases' action, which leads to changes in protein size, structure and hydrophobicity, as well as changes in its techno-biofunctional properties (Kleekayai & FitzGerald 2022). MPH are mainly designed for nutritional approaches, such as sports nutrition, enteral formulas and hypoallergenic infant formulas due to their high nutritional value, amino acid composition, good digestibility, commercial availability and moderate cost (Clemente, 2000; Singh & Ye 2014).

Enzymatic hydrolysis modulates and potentializes milk protein features, such as viscosity, solubility, foaming, emulsification, among others; besides, it provides advantages in food application as ingredient (De Castro et al., 2015). Banach et al. (2013) investigated the functional properties of MPC 80 by using different peptidases in enzymatic hydrolysis. Hydrolysates have shown increased solubility, likely due to the reduced number of hydrophobic functional groups on the protein surface, which presents better solubility in water. Emulsification capacity was also enhanced after hydrolysis, mainly due to the incidence of smaller peptides with hydrophobic and hydrophilic features (Banach et al., 2013). The same study highlighted enzymes' ability to hydrolyze caseins and whey proteins. Although all enzymes (trypsin, chymotrypsin, pepsin and papain) were capable of hydrolyzing caseins, only chymotrypsin and papain have hydrolyzed whey proteins. According to the aforementioned authors, this effect was justified by structural differences among milk proteins, since the flexible random structure of caseins is more easily hydrolyzed by peptidases than the globular forms of whey proteins (β-lactoglobulin and α-lactalbumin) (Banach et al., 2013; Guo et al., 1995).

These techno-functional properties of MPH are also influenced by post thermal hydrolysis treatments. Amigo-Benavent and FitzGerald (2022) have assessed the effect of thermal enzyme inactivation conditions on WPC hydrolysates' viscosity and gelation trend. Their findings have evidenced that WPH viscosity values were 16% and 18% lower than those recorded for unhydrolyzed WPC, since peptides released by enzymatic hydrolysis have smaller molecular size and lesser secondary structures than native proteins. However, WPC hydrolysates presented increased viscosity after heating due to growing particle-particle interaction between smaller peptides, a fact that indicated aggregate formations (Amigo-Benavent & FitzGerald, 2022).

### 3.3.5 Bioactive Peptides

In addition, casein (α- β- and κ-casein) and whey proteins (α-lactalbumin, β-lactoglobulin, bovine serum albumin, and lactoferrin) hydrolyzed by peptidases can release expressive amounts of bioactive peptides (Korhonen, 2006). These compounds are short protein fragments that interact with appropriate cellular receptors and regulate physiological functions in the human body. Some peptides deriving from milk protein hydrolysis have been associated with antioxidant, antibacterial, antifungal, antiviral, ACE-inhibitory, immunomodulating, antiproliferative, antithrombotic, and anticoagulant properties in the human organism (Table 3). Bioactive peptides often have 3–16 amino acid residues, and their activity is based on their amino acid composition and sequence (Ryhänen et al., 2001; Korhonen, 2009). Therefore, their biological activity results from the profiling of several released peptide fragments, whose specific peptide sequences may reveal two or more different biological activities (Zanutto-Elgui et al., 2019).

**Table 3** - Bioactive peptides released from milk proteins enzymatic hydrolysis.

| Enzyme Used | Bioactivity | Characteristic of the identified bioactive peptide | Substrate | Reference |
|---|---|---|---|---|
| Bacterial enzymes | Opioid activity, ACE inhibitory | Ser-Lys-Val-Tyr-Pro β-casein f(60–66) | Enzyme-Modified Cheese | Haileselassie et al, (1999) |
| Neutrase | ACE inhibitory and antioxidant | Fractions with 0.2–3.5 kDa (ACE inhibitory) and 8–3.5 kDa (Antioxidant) | Milk protein concentrate | Uluko et al, (2014) |
| Commercial peptidases | Antioxidant | Low molecular weight peptides (<3 kDa), from αs1-casein, β-casein, κ-casein, and αs2-casein. | Milk protein concentrate | Cui et al, (2022) |
| Pepsin and neutral protease | Antioxidant and ACE inhibitory | Low molecular weight peptides | Caprine milk proteins | Koirala et al, (2021) |
| Pepsin and pancreatin | Antioxidant and ACE inhibitory | Fractions with less than 10 kDa | Bovine colostrum whey | Espinoza et al, (2020) |
| Bacterial peptidase | ACE inhibitory and antimicrobial | ACE-inhibitory peptides from β-LG f(48–56), antimicrobial from β-LG f(41–56) | Whey protein | Worsztynowicz et al, (2020) |
| Fungal peptidases | Antioxidant and Antimicrobial | peptides deriving from α-s1-casein | Bovine and goat milk | Zanutto-Elgui et al, (2019) |
| Pepsin | Antioxidant | Peptide fractions (>10 kDa) | Whey protein concentrate | Alizadeh and Aliakbarlu (2020) |
| Trypsin and flavourzyme | Antioxidant | Peptides deriving from β-casein, fractions up to >2 kDa | Bovine milk casein | Bamdad et al, (2017) |
| Pepsin, trypsin, chymotrypsin | Antioxidant | Low molecular weight peptides (≤1 kDa) from αS1-casein, αS2-casein, β-casein and κ-casein | Buffalo casein | Shanmugam et al, (2015) |
| Alcalase | Antioxidant | Low molecular weight | Cheese whey | Athira et al, (2014) |

| | | peptides (<3 kDa) from β-Lg (96–100), (123–134), (122–131) | | |
|---|---|---|---|---|
| Commercial proteases | Antioxidant | Low molecular weight peptides (≤1 kDa) | Goat milk protein | De Gobba *et al,* (2014) |
| Animal, plant and microbial peptidase | Antioxidant | Low molecular weight peptides (<3 kDa) derived from β-casein and αs1-casein | Hard cow milk cheese | Timón *et al,* (2014) |
| Alcalase | Antioxidant | Low molecular weight peptides | Whey protein concentrate | Lin *et al,* (2012) |
| Pancreatic enzyme and thermolysin | Antioxidant | Peptides LQKW f (58–61) and LDTDYKK f(95–101) from β-Lg | Whey protein concentrate | Contreras *et al,* (2011) |
| Proteinase K, trypsin, pepsin and *Bacillus licheniformis* peptidase | ACE-inhibitory activity | β-casein f100–105 and α-La f21–26 and f15–26 peptides | Casein and whey proteins | Otte *et al,* (2007) |
| Trypsin and chymotrypsin | Antibacterial | Polypeptides linked by a disulfide bridge | Bovine α-lactalbumin molecule | Pellegrini (1999) |
| Peptidases from Lactic acid bacteria strains | ACE-inhibitory and immunomodulatory | Peptide fractions (<10 kDa) | Milk fermentation cultures | Adams *et al,* (2020) |
| Trypsin, chymotrypsin, proteinase K and thermolysin | ACE-inhibitory | Fragments (46–53), f(58–61), (103–105), and (122–125), from β-Lg | Caprine β-Lg preparations | Hernández-Ledesma *et al,* (2002) |
| Bacterial peptidases | ACE-inhibitory | Fragments from αs1-cn (1–9), (1–7), (1–6) | Ripened cheese | Ryhänen *et al,* (2001) |
| Bacterial enzymes | Opioid activity, ACE inhibitory | Ser-Lys-Val-Tyr-Pro β-casein f(60–66) | Enzyme-Modified Cheese | Haileselassie *et al,* (1999) |
| Pepsin | Opioid | Sequence of the amino acids Tyr-Gly-Leu-Phe (f50–53) | Bovine α-Lactalbumin | Horikawa *et al,* (1983) |
| Enzyme no mentioned | Opioid | β-casomorphins peptides | Casein-derived peptides | Brantl *et al,* (1981) |

Source: Authors.

Bioactive peptides released through enzymatic hydrolysis depend on enzyme type, as well as on hydrolysis conditions, such as enzyme-substrate concentration, temperature and pH (Shivanna & Nataraj 2020). Each enzyme has a specific catalytic action site; thus, peptidases used in hydrolysis processes have direct influence on the release of milk-deriving bioactive peptides. For instance, pepsin, trypsin and chymotrypsin-based enzymatic hydrolysis of milk proteins released several antihypertensive peptides, calcium-binding phosphopeptides, as well as antibacterial, immunomodulatory and opioid peptides, both from casein fractions and whey proteins (Korhonen, 2009).

Moreover, plant peptidases (deriving from melon fruit, trompillo berries and citrus flowers) have shown potential to be used to produce whey protein hydrolysates with bioactive properties (Mazorra-Manzano et al., 2020). Concerning plant peptidases, papain and bromelain-like cysteine peptidases have been mostly explored to generate bioactive peptides (Mazorra-Manzano et al., 2017).

Another strategy adopted to generate bioactive peptides lies on fermenting milk protein substrates by using proteolytic microorganisms, such as Lactobacillus, Streptococcus, Staphylococcus and Bacillus strains (Cavalheiro et al., 2020; Leclerc et al., 2002; Nielsen et al., 2022; Skrzypczak et al., 2020). For instance, milk proteins are hydrolyzed by the action of peptidases secreted through supplemented microorganisms during the development of fermented milk products (Nasri et al., 2022).

**3.3.6 Emergent technologies focused on improving enzymatic hydrolysis**

Given the diversity of likely peptidase applications in the dairy industry, researchers have been investigating alternatives to improve the action of these enzymes based on pre-treatment application on dairy substrates, for example. Using advanced technologies to pretreat milk proteins can enhance hydrolysates' biological properties, enable peptidases' action on active substrates and, consequently, improve the hydrolysis process (Alizadeh & Aliakbarlu 2020; Uluko et al., 2014). Therefore, different thermal and nonthermal treatments, and their synergic effects, have been investigated.

Athira et al. (2014) have used preheating treatment to optimize hydrolysis conditions adopted to develop whey protein hydrolysates (WPH). According to the aforementioned authors, the preheating process leads to slight whey protein denaturation, which enables peptidase action and helps improving enzymatic hydrolysis (Athira et al., 2014). On the other hand, Mikhaylin et al. (2017) have shown that high voltage electrical treatments have improved trypsin-based β-lactoglobulin hydrolysis by up to 66% and enhanced hydrolysis degree by 80%.

Alizadeh and Aliakbarlu (2020) have investigated the use of combined treatments and noticed that ultrasound and ohmic heating pretreatments have significantly increased the hydrolysis degree and antioxidant activity of whey protein concentrate hydrolysates. Ultrasound effect on the physicochemical properties of protein molecules is associated with cavitation, whereas ohmic heating is in line with the passing of electrical current through food products, which converts electrical energy into heat (Alizadeh & Aliakbarlu 2020; Sakr & Liu 2014). Likewise, Uluko et al. (2015) have shown that using heating, microwave and ultrasound as pretreatments has increased antioxidative peptides' release from pepsin and trypsin-based MPC hydrolysis. Recent studies, such as the one conducted by Koirala et al. (2021), have also shown positive results associated with ultrasonic effects before enzymatic hydrolysis of caprine milk protein. Moreover, El Mecherf et al. (2011) have successfully enhanced β-lactoglobulin hydrolysis based on using microwave as pretreatment, which enabled the release of low-immunoreactivity hydrolysates (El Mecherf et al. 2011; El Mecherfi et al. 2014).

Other authors have investigated the use of high pressure to optimize hydrolysis conditions, since it may reduce enzymatic hydrolysis reaction time, increase hydrolysates, as well as improve the availability of bioactive peptides deriving from milk proteins (Bamdad et al., 2017; Barba et al., 2015). High-pressure homogenization (HPH) at 100–200 MPa has induced structure unfolding on bovine serum albumin protein, which increased trypsin and chymotrypsin action, and accelerated the enzymatic hydrolysis reaction rate (Carullo et al., 2020). Previous studies, such as the one conducted by Blayo et al. (2016), have used HPH treatment (300 MPa) to accelerate enzymatic hydrolysis reaction rates. Results in the aforementioned studies were attributed to partial whey proteins' unfolding, which increased the accessibility on active substrates for trypsin hydrolysis (Blayo et al., 2016).

## 4. Conclusions

Commercial peptidases constitute a consolidated market with potential to grow in the dairy sector. These enzymes have wide specificity, which enables obtaining hydrolysates with different functional and nutritional features. Recent scientific studies conducted with peptidases used for dairy production purposes have focused on finding alternatives to improve peptidases' action in milk proteins, as well as on investigating new enzymes capable of meeting the demands of the dairy industry. However, it is necessary to conduct innovative studies to help better understanding peptidases' action in both caseins and whey proteins, to enable hydrolysis processes and achieve the intended hydrolysates, as well as to expand their applications in dairy.

## Acknowledgments

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