VIRGÍNIA NARDY PAIVA

ENZYMATIC HYDROLYSIS OF MILK PROTEINS: OPTIMIZATION FOR OBTAINING BIOACTIVE HYDROLYSATES

Thesis submitted to the Graduate Program in Food Science and Technology of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Antônio Fernandes de Carvalho

Co-advisors: Evandro Martins
Solimar Gonçalves Machado

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ABSTRACT

PAIVA, Virgínia Nardy, D.Sc., Universidade Federal de Viçosa, November, 2022. **Enzymatic hydrolysis of milk proteins: Optimization for obtaining bioactive hydrolysates.** Adviser: Antônio Fernandes de Carvalho. Co-advisors: Evandro Martins and Solimar Gonçalves Machado.

Enzymatic hydrolysis modifies the physical structure of milk proteins and promotes changes in their technological and biofunctional properties. The protein source, the proteolytic enzyme used, and the hydrolysis conditions employed determine the resulting characteristics of the hydrolyzate (eg extent of hydrolysis, peptide profile, and techno- and biofunctional properties). In this sense, the first chapter approaches the main characteristics of the enzymes applied in the hydrolysis of milk proteins (peptidases) and also mentions their applications in the dairy industry, from the use of peptidases in the manufacture of cheese to the hydrolysis of milk proteins for generation of bioactive compounds. Following this theme, the second chapter presents the use of commercial peptidases in the enzymatic hydrolysis of whey proteins aiming for antioxidant hydrolysates released. In this study, the response surface methodology was used to optimize and maximize the hydrolysis degree and antioxidant activity responses. The hydrolysates showed a maximum antioxidant activity of 13.7% by the ABTS methodology, and the highest degree of hydrolysis of 5.16% was obtained by evaluating the soluble peptides in trichloroacetic acid. In general, the objective of this proposal was to present the different strategies for the commercial use of peptidases in dairy technology with a focus on optimizing the hydrolysis conditions of whey proteins to obtain bioactive compounds.

Keywords: Commercial enzymes. Enzymatic hydrolysis. Whey protein hydrolysates. Bioactive peptides. Antioxidant activity.

RESUMO

PAIVA, Virgínia Nardy, D.Sc., Universidade Federal de Viçosa, novembro de 2022. **Hidrólise enzimática das proteínas do leite: otimização para obtenção de hidrolisados bioativos.** Orientador: Antônio Fernandes de Carvalho. Coorientadores: Evandro Martins e Solimar Gonçalves Machado.

A hidrólise enzimática modifica a estrutura física das proteínas do leite e promove mudanças em suas propriedades tecnológicas e biofuncionais. A fonte proteíca, a enzima proteolítica utilizada e as condições de hidrólise empregadas ditam as características resultantes do hidrolisado (por exemplo, a extensão da hidrólise, perfil peptídico e as propriedades tecno e biofuncionais). Neste sentido, o primeiro capítulo desta tese aborda as principais características das enzimas aplicadas na hidrólise das proteínas do leite (peptidases) e cita suas principais aplicações na indústria de laticínios, desde a utilização de peptidases na fabricação de queijos até a hidrólise de proteínas lácteas para geração de compostos bioativos. Dentro deste útilmo tema, o segundo capítulo apresenta a utilização de peptidases comerciais na hidrólise enzimática de proteínas do soro de leite, com o objetivo da geração de hidrolisados com propriedades antioxidantes. Neste estudo, foi empregada a metodologia de superfície de resposta visando a otimização e maximização das respostas de grau de hidrólise e atividade antioxidante. Os hidrolisados apresentaram máxima atividade antioxidante de 13.7% utilizando a metodologia ABTS, e o maior grau de hidrólise de 5.16%, obtido através da avaliação de peptídeos solúveis em ácido tricloroacético. De maneira geral, o objetivo desta proposta foi apresentar as diferentes estratégias no uso de peptidases comerciais na tecnologia de latícinios com foco na otimização das condições de hidrólise de proteínas do soro do leite a fim da obtenção de compostos bioativos.

Palavras-chave: Enzimas comerciais. Hidrólise enzimática. Hidrolisados de proteína de soro de leite. Peptídeos bioativos. Atividade antioxidante.

CONTENTS

1. General Introduction

Dairy products are an important source of high-value proteins, composed of caseins (80%) and whey proteins (20%) (Walstra et al., 2005). Casein consists of four fractions, i.e., αS1, αS2-, β- and κ-casein, and they presented as large structures known as casein micelles (Fox & Mulvihill,1982; Hazlett et al., 2018). Whey proteins are soluble proteins with a tertiary structure, mainly comprised of β lactoglobulin (β-Lg), α-lactalbumin (α-La), bovine serum albumin (BSA), and immunoglobulin (Fox & Mulvihill,1982; Wijayanti et al., 2019).

The industrial uses of milk proteins are based on their unique composition, functionality, and nutritive values, as well as they, have been recognized as one of the main sources of biologically active peptides (Hazlett et al., 2018; Mann et al., 2019). These include opioid agonist and antagonist peptides, potential hypotensive peptides which inhibit an angiotensin-I-converting enzyme (ACE), antibacterial, anticarcinogenic and antioxidant peptides (Fitzgerald & Meisel, 2003; Nongonierma et al., 2016; Dullius et al., 2018; Mann et al., 2019; Bielecka et al., 2022).

The bioactive peptide sequences are encrypted within their milk protein primary structures, which can be released through enzymatic hydrolysis (PihlantoLeppälä, 2000). Peptidases are hydrolase enzymes able to catalyze the hydrolysis of proteins' peptide bonds (Gurumallesh et al., 2019). These enzymes have different substrate specificities, diversity of active sites, catalytic mechanisms, optimum pHs, optimum temperatures, and stability profiles (MartínezMedina et al., 2019). They have been currently used as food-grade enzymes and others are being researched for the production of milk protein hydrolysates with tailored functionality and biological activity (Jeewanthi et al., 2015).

Through the diversity of milk proteins, the wide variety of peptidases, and the different hydrolysis conditions used, such as temperature and reaction time, several hydrolysates could be released for different biotechnological applications (Abd ElSalam & El-Shibiny, 2015). In this context, chapter 1 “Peptidases used in dairy technology: Current knowledge and relevant applications”, approached the main peptidases applications in the dairy industry, and some important aspects of their biotechnological use. Whereas, chapter 2 the paper “Hydrolysis of whey protein and antioxidant activity of hydrolysates: Optimization by response surface methodology” presented a studied for the optimization of hydrolysis conditions aimed at the release of whey protein hydrolysates with antioxidant properties. These studies aimed to present the general use of commercial peptidases in dairy technology, focusing on improving enzymatic hydrolysis conditions to release bioactive compounds.

References

Abd El-Salam, M. H., & El-Shibiny, S. (2015). Preparation, properties, and uses of enzymatic milk protein hydrolysates. Critical Reviews in Food Science and Nutrition, 57(6), 1119–1132. doi:10.1080/10408398.2014.899200

Bielecka, M., Cichosz, G., & Czeczot, H. (2022). Antioxidant, antimicrobial and anticarcinogenic activities of bovine milk proteins and their hydrolysates - A review. International Dairy Journal, 127, 105208. https://doi.org/10.1016/j.idairyj.2021.105208

Dullius, A., Goettert, M. I., & de Souza, C. F. V. (2018). Whey protein hydrolysates as a source of bioactive peptides for functional foods – Biotechnological facilitation of industrial scale-up. Journal of Functional Foods, 42, 58-74. https://doi.org/10.1016/j.jff.2017.12.063

Fitzgerald, R. J., & Meisel, H. (2003). Milk Protein Hydrolysates and Bioactive Peptides. Advanced Dairy Chemistry—1 Proteins, 675–698. doi:10.1007/978-1-4419-8602-3_20

Fox, P. F., & Mulvihill, D. M. (1982). Milk proteins: molecular, colloidal and functional properties. Journal of Dairy Research, 49(04), 679. doi:10.1017/s0022029900022822

Gurumallesh, P., Alagu, K., Ramakrishnan, B., & Muthusamy, S. (2019). A systematic reconsideration on proteases. International Journal of Biological Macromolecules. doi:10.1016/j.ijbiomac.2019.01.08

Hazlett, R., Schmidmeier, C., & O'Mahony, J. A. (2018). Encyclopedia of Food Chemistry: Milk Proteins. Reference Module in Food Science. doi:10.1016/b978-0-08-100596-5.21604-1

Jeewanthi, R. K. C., Lee, N.-K., & Paik, H.-D. (2015). Improved Functional Characteristics of Whey Protein Hydrolysates in Food Industry. Korean Journal for Food Science of Animal Resources, 35(3), 350–359. doi:10.5851/kosfa.2015.35.3.350

Mann, B., Athira, S., Sharma, R., Kumar, R., & Sarkar, P. (2019). Bioactive Peptides from Whey Proteins. Whey Proteins, 519–547. doi:10.1016/b978-0-12-812124-5.00015-1

Nongonierma, A. B., O'Keeffe, M. B., & FitzGerald, R. J. (2016). Milk Protein Hydrolysates and Bioactive Peptides. Advanced Dairy Chemistry, 417–482. doi:10.1007/978-1-4939-2800-2_15

Walstra, P., Walstra, P., Wouters, J.T.M., & Geurts, T.J. (2005). Dairy Science and Technology (2nd ed.). CRC Press. https://doi.org/10.1201/9781420028010

Wijayanti, H. B., Brodkorb, A., Hogan, S. A., & Murphy, E. G. (2019). Thermal Denaturation, Aggregation, and Methods of Prevention. Whey Proteins, 185–247. doi:10.1016/b978-0-12-812124-5.00006-0

2. CHAPTER 1

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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.

Peptidases used in dairy technology: Current knowledge and relevant applications

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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.

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	Protamex®	Dairy protein hydrolysate
(Brazil)	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.

Methodology

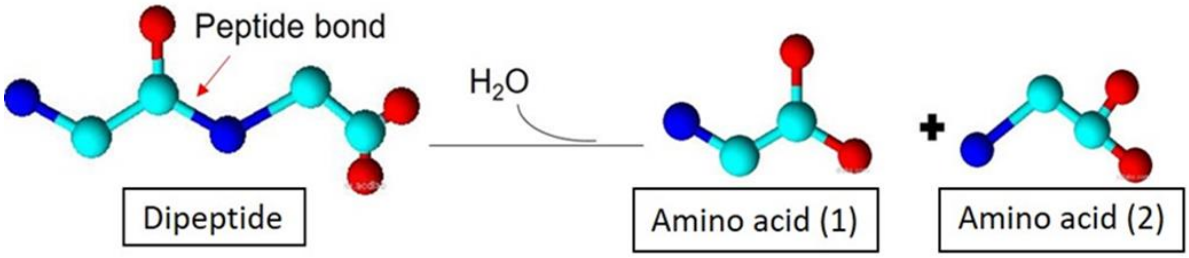
This review was based on the research of scientific articles from different indexing bases, regarding the main characteristics of peptidases and their biotechnological applications in dairy products. The papers adopted for the construction and discussion of this review include the most relevant and current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

Results and Discussion

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.

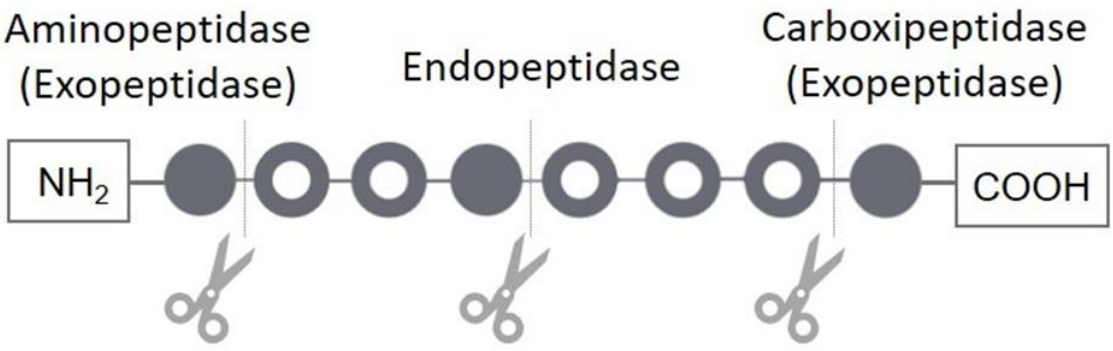


Source: Adapted from Mótyán et al. (2013)

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, metallo carboxypeptidases, 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	2 – 3	Papain and bromelain

			mercury benzoic acid		
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).

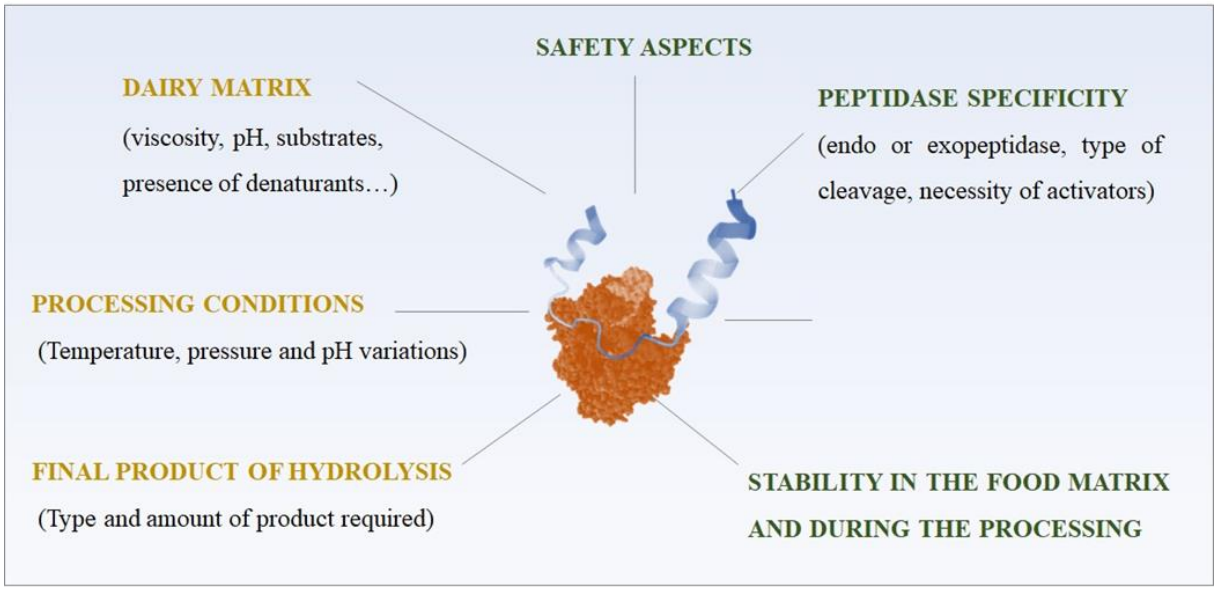
Requirements for peptidase application in the dairy industry

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

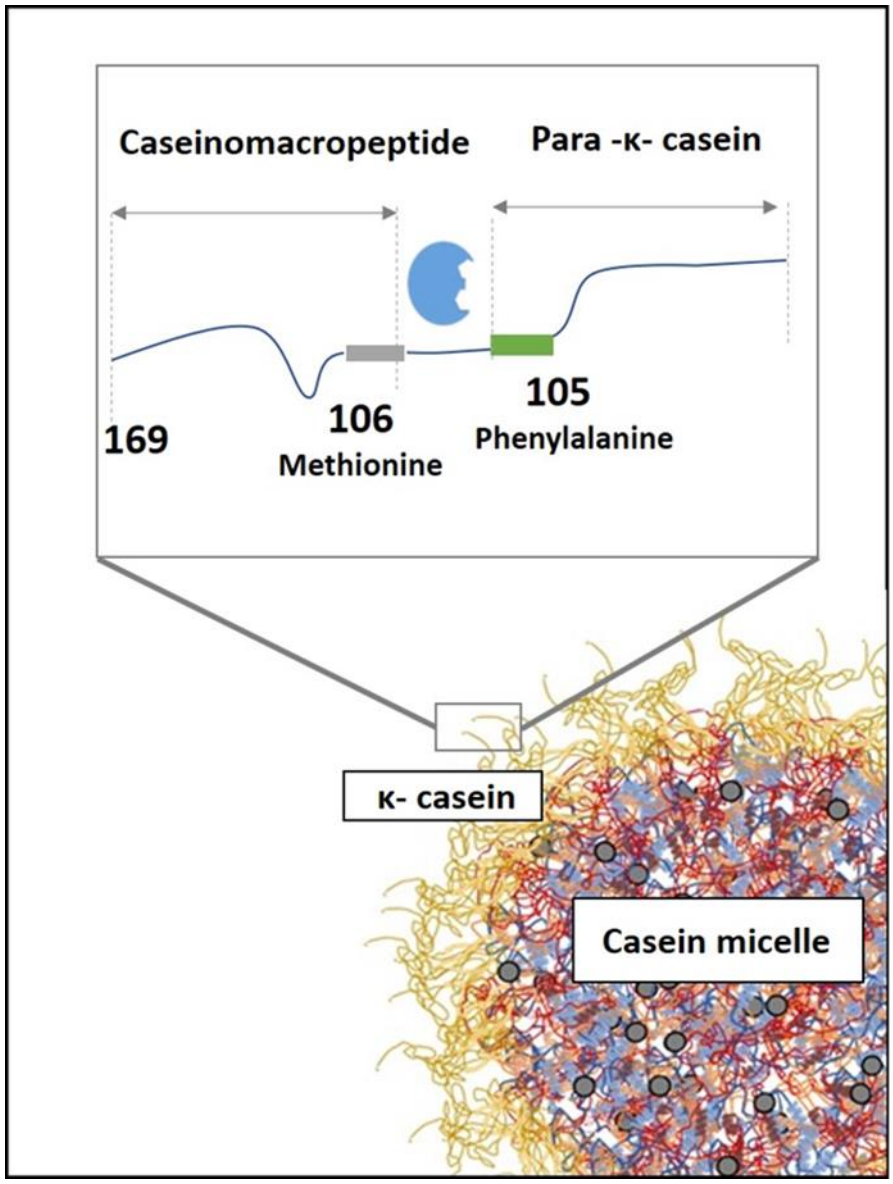
Main peptidase applications in the dairy industry

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).

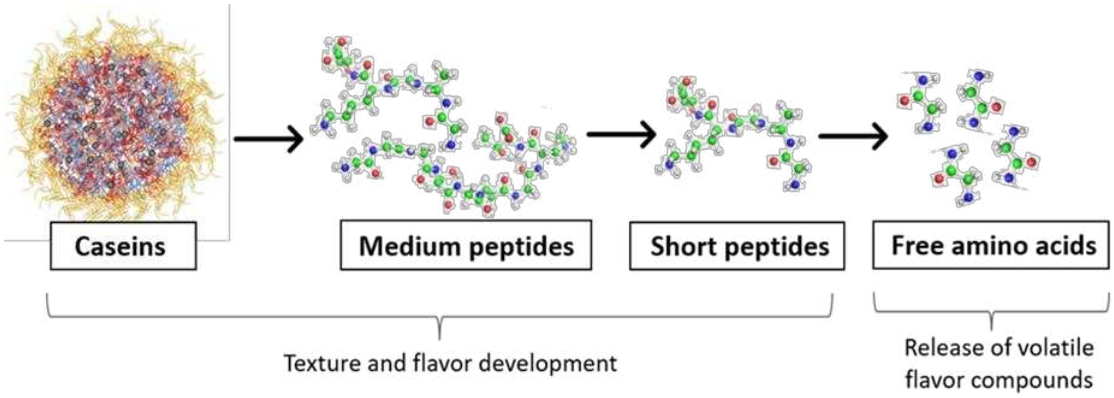
The increasing demand observed in the cheese market, in association with expansive costs with animal rennet and with religious or dietary concerns about its consumption, have prompted the use of alternative peptidase sources with coagulant properties (Lemes et al., 2016; Zikiou & Zidoune, 2018). These 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).

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, 2000). 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,

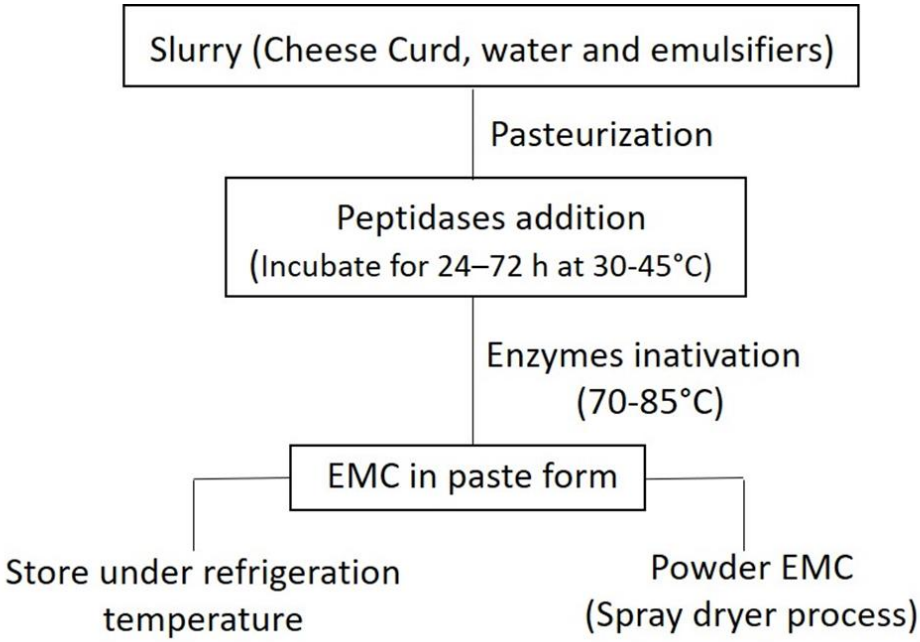
it is important taking into consideration the safety and suitability of the encapsulation material for large-scale production.

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).

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).

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 peptides (<3 kDa) from β-Lg (96–100), (123–134), (122–131)	Cheese whey	Athira et al, (2014)
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).

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, 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 improve 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).

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.

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References

Abada, E. A. (2019). Application of Microbial Enzymes in the Dairy Industry. In Kuddus, M (Ed.), Enzymes in Food Biotechnology - Production, Applications, and Future Prospects (pp.61-72). Academic Press, Elsevier Inc. https://doi.org/10.1016/B978-0-12-813280-7.00005-0

Afsharnezhad, M., Shirin, S., & Sariri, R. (2018). A novel milk-clotting cysteine protease from *Ficus johannis*: Purification and characterization. International Journal of Biological Macromolecules, 121, 173-182. https://doi.org/10.1016/j.ijbiomac.2018.10.006

Agudelo, R. A., Gauthier, S. F., Pouliot, Y., Marin, H., & Savoie, L. (2004). Kinetics of peptide fraction release during in vitro digestion of casein. Journal of the Science of Food and Agriculture, 84 325–333. https://doi.org/10.1002/jsfa.1662

Ahmed, I. A. M., Morishima, I., Babiker, E. E., & Mori, N. (2009). Characterisation of partially purified milk-clotting enzyme from *Solanum dubium* Fresen seeds. Food Chemistry, 116 395–400. https://doi.org/10.1016/j.foodchem.2008.11.072

Ahmed, S. A., Wehaidy, H, R., Ibrahim, O, A., Abd El Ghani, S., & El-Hofi, M. A. (2016). Novel milk-clotting enzyme from *Bacillus stearothermophilus* as a coagulant in UF-white soft cheese. Biocatalysis and Agricultural Biotechnology, 7 241–249. https://doi.org/10.1016/j.bcab.2016.06.011

Alavi, F., & Momen, S. (2020). Aspartic proteases from thistle flowers: traditional coagulants used in the modern cheese industry. International Dairy Journal, 107104709. https://doi.org/10.1016/j.idairyj.2020.104709

Ali, B., Khan, K. Y., Majeed, H., Jin, Y., Xu, D., Rao, Z., & Xu, X. (2022). Impact of Soy–Cow's mixed milk enzyme modified cheese on bread aroma. LWT-Food Science and Technology 154 112793. https://doi.org/10.1016/j.lwt.2021.112793

Alizadeh, O., & Aliakbarlu, J. (2020). Effects of ultrasound and ohmic heating pretreatments on hydrolysis, antioxidant and antibacterial activities of whey protein concentrate and its fractions. LWT-Food Science and Technology, 131 109913. https://doi.org/10.1016/j.lwt.2020.109913

Athira, S., Mann, B., Saini, P., Sharma, R., Kumar, R., & Singh, A. K. (2014). Production and characterisation of whey protein hydrolysate having antioxidant activity from cheese whey. Journal of the Science of Food and Agriculture, 95 2908–2915. https://doi.org/10.1002/jsfa.7032

Azarnia, S., Lee, B., St-Gelais, D., Kilcawley, K., & Noroozi, E. (2011). Effect of free and encapsulated recombinant aminopeptidase on proteolytic indices and sensory characteristics of Cheddar cheese. LWT - Food Science and Technology, 44 570–575. https://doi.org/10.1016/j.lwt.2010.08.022

Bamdad, F,. Shin, S. H., Suh, J-W., Nimalaratne, C., & Sunwoo, H. (2017). Anti-Inflammatory and Antioxidant Properties of Casein Hydrolysate Produced Using High Hydrostatic Pressure Combined with Proteolytic Enzymes. Molecules, 22 609. https://doi.org/10.3390/molecules22040609

Banach, J. C., Lin, Z., & Lamsal, B. P. (2013). Enzymatic modification of milk protein concentrate and characterization of resulting functional properties. LWT Food Science and Technology, 54 397-403. https://doi.org/10.1016/j.lwt.2013.06.023

Barba, F. J., Terefe, N. S., Buckow, R., Knorr, D., & Orlien, V. (2015). New opportunities and perspectives of high-pressure treatment to improve health and safety attributes of foods. A review. Food Research International, 77 725–742. https://doi.org/10.1016/j.foodres.2015.05.015

Barrett, A. J., & McDonald, J. K. (1986). Nomenclature: protease, proteinase and peptidase. Biochemical Journal, 237 935–935. https://doi.org/10.1042/bj2370935

Barrett, A. J. (1999). Peptidases: a view of classification and nomenclature. In: Turk V. (eds) Proteases New Perspectives. MCBU Molecular and Cell Biology Updates.

Barrett, A. J. (2000). Proteases. Current Protocols in Protein Science, 21(1). doi:10.1002/0471140864.ps2101s21

Bas, D., Kendirci, P., Salum, P., Govce, G., & Erbay, Z. (2019). Production of enzyme-modified cheese (EMC) with ripened white cheese flavour: I-effects of proteolytic enzymes and determination of their appropriate combination. Food and Bioproducts Processing, 117 287-301. https://doi.org/10.1016/j.fbp.2019.07.016

Biocatalysts. (2015). available at https://www.biocatalysts.com/?s=Promod%E2%84%A2+215MDP accessed on March 10, 2022

Blayo, C., Vidcoq, O., Lazennec, F., & Dumay, E. (2016). Effects of high pressure processing (hydrostatic high pressure and ultra-high pressure homogenisation) on whey protein native state and susceptibility to tryptic hydrolysis at atmospheric pressure. Food Research International, 79 40–53. https://doi.org/10.1016/j.foodres.2015.11.024

Carullo, D., Donsì, F., & Ferrari, G. (2020). Influence of high-pressure homogenization on structural properties and enzymatic hydrolysis of milk proteins. LWT Food Science and Technology, 130 109657. https://doi.org/10.1016/j.lwt.2020.109657

Cavalheiro, G. F., Parra Baptista, D., Domingues Galli, B., Negrão, F., Nogueira Eberlin, M., & Lúcia Gigante, M. (2020). High protein yogurt with addition of *Lactobacillus helveticus*: Peptide profile and angiotensin-converting enzyme ACE-inhibitory activity. Food Chemistry, 333, 127482. doi:10.1016/j.foodchem.2020.12748

Cavalcanti, M., Teixeira, M. F. S., Lima Filho, J. L., & Porto, A. L. F. (2004). Partial purification of new milk-clotting enzyme produced by *Nocardiopsis* sp. Bioresource Technology, 93 29–35. https://doi.org/10.1016/j.biortech.2003.10.003

Cagno, D. R., Pasquale, I., Angelis, M., & Gobbetti, M. (2012). Accelerated ripening of Caciocavallo Pugliese cheese with attenuated adjuncts of selected nonstarter lactobacilli. Journal of Dairy Science, 95 4784–4795. https://doi.org/10.3168/jds.2011-5283

Cavalli, V. S., Lufrano, D., Colombo, M. L., & Priolo, N. (2013). Properties and applications of phytepsins from thistle flowers. Phytochemistry, 92 16–32. https://doi.org/10.1016/j.phytochem.2013.04.013

Clemente, A. (2000). Enzymatic protein hydrolysates in human nutrition. Trends in Food Science & Technology, 11 254–262. https://doi.org/10.1016/S0924-2244(01)00007-3

Cui, Q., Sun, Y., Cheng, J., & Guo, M. (2022). Effect of two-step enzymatic hydrolysis on the antioxidant properties and proteomics of hydrolysates of milk protein concentrate. Food Chemistry, 366 130711. https://doi.org/10.1016/j.foodchem.2021.130711

De Castro, R. J. S., Bagagli, M. P., & Sato, H. H. (2015). Improving the functional properties of milk proteins: focus on the specificities of proteolytic enzymes. Current Opinion in Food Science, 1 64–69. https://doi.org/10.1016/j.cofs.2014.12.004

Dhillon, A., Sharma, K., Rajulapati, V., & Goyal, A. (2017). Proteolytic Enzymes. Current Developments in Biotechnology and Bioengineering, 149–173. https://doi.org/10.1016/B978-0-444-63662-1.00007-5

Dupas, C., Métoyer, B., Hatmi, H. E., Adt, I., Mahgoub, S. A., & Dumas, E. (2020). Plants: A natural solution to enhance raw milk cheese preservation. Food Research International, 130 108883. https://doi.org/10.1016/j.foodres.2019.108883

Ehrmann, M., & Clausen, T. (2004). Proteolysis as a Regulatory Mechanism. Annual Review of Genetics, 38 709–724. https://doi.org/10.1146/annurev.genet.38.072902.093416

El Mecherfi, K. E., Saidi, D., Kheroua, O., Boudraa, G., Touhami, M., Rouaud, O., Curet, S., Choiset, Y., Rabesona, H., Chobert, J-M., & Haertlé, T. (2011). Combined microwave and enzymatic treatments for β-lactoglobulin and bovine whey proteins and their effect on the IgE immunoreactivity. European Food Research and Technology, 233 859–867. https://doi.org/10.1007/s00217-011-1581-y

El Mecherfi, K-E., Rouaud, O., Cure, S., Negaoui, H., Chobert, J-M., Kheroua, O., Saidi, D., & Haertlé, T. (2014). Peptic hydrolysis of bovine beta-lactoglobulin under microwave treatment reduces its allergenicity in anex vivomurine allergy model. International Journal of Food Science & Technology, 50 356–364. https://doi.org/10.1111/ijfs.12653

Food and Agriculture Organization. (2021). https://www.fao.org/food/food-safety-quality/scientific-advice/jecfa/jecfa-additives/enzymes/en/. Accessed on March 04, 2022 Fox, P. F., O'Connor, T., Mcsweeney, P., Guinee, T., O'brien, N. (1996). Cheese:

Physical, biochemical, and nutritional aspects. Advances in Food and Nutrition Research, 39 163–328. https://doi.org/10.1016/S1043-4526(08)60075-3

Gagaoua, M., Hoggas, N., & Hafid, K. (2015). Three phase partitioning of zingibain, a milk-clotting enzyme from *Zingiber officinale* Roscoe rhizomes. International Journal of Biological Macromolecules, 73 245–252. https://doi.org/10.1016/j.ijbiomac.2014.10.069

Gagaoua, M., Hafid, K., & Hoggas, N. (2017). Data in support of three phase partitioning of zingibain, a milk-clotting enzyme from *Zingiber officinale* Roscoe rhizomes. Data in Brief, 6 634–639. https://doi.org/10.1016/j.dib.2016.01.014

Gagaoua, M., Ziane, F., Nait Rabah, S., Boucherba, N., El-Hadef El-Okki, A., Bouanane-Darenfed, A., & Hafid, K. (2017). Three phase partitioning, a scalable method for the purification and recovery of cucumisin, a milk-clotting enzyme, from the juice of *Cucumis melo* var . reticulatus. International Journal of Biological Macromolecules, 102 515–525. https://doi.org/10.1016/j.ijbiomac.2017.04.060

Garcia, H. S., López-Hernandez, A., & Hill, C. G. (2017). Enzyme Technology – Dairy Industry Applications. Reference Module in Life Sciences. https://doi.org/10.1016/B978-0-12-809633-8.09232-3

Gomes, H. A. R., Moreira, L. R. S., & Filho, E. X. F. (2018) Enzymes and Food Industry: A Consolidated Marriage. Advances in Biotechnology for Food Industry, 55–89. https://doi.org/10.1016/B978-0-12-811443-8.00003-7

Güler, G., Vorob'ev, M. M., Vogel, V., & Mäntele, W. (2016). Proteolytically-induced changes of secondary structural protein conformation of bovine serum albumin monitored by Fourier transform infrared (FT-IR) and UV-circular dichroism spectroscopy. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 161 8–18. https://doi.org/10.1016/j.saa.2016.02.013

Guleria, S., Walia, A., Chauhan, A., & Shirkot, C. K. (2016). Optimization of milk-clotting enzyme production by *Bacillus amyloliquefaciens* SP1 isolated from apple rhizosphere. Bioresources and Bioprocessing, 3 30. https://doi.org/10.1186/s40643-016-0108-6

Guo, M. R., Fox, P. F., Flynn, A., & Kindstedt, P. S. (1995). Susceptibility of β-Lactoglobulin and Sodium Caseinate to Proteolysis by Pepsin and Trypsin. Journal of Dairy Science, 78 2336–2344. https://doi.org/10.3168/jds.S0022-0302(95)76860-6

Gurumallesh, P., Alagu, K., Ramakrishnan, B., & Muthusamy, S. (2019). A systematic reconsideration on proteases. International Journal of Biological Macromolecules, 1 128-254. https://doi.org/10.1016/j.ijbiomac.2019.01.081

Gurung, N., Ray, S., Bose, S., & Rai, V. (2013). A Broader View: Microbial Enzymes and Their Relevance in Industries, Medicine, and Beyond. BioMed Research International, 13 1-18. https://doi.org/10.1155/2013/329121 Gripon, J. C., Monnet, V., Lambert, G., & Desmazeaud, M. J. (1991). Microbial enzymes in cheese ripening Food enzymology . In P.F. Fox (Ed.), Elsevier Applied Science (pp.131-168).

Grozdanovic, M. M., Burazer, L., & Gavrovic-Jankulovic, M. (2013). Kiwifruit (*Actinidia deliciosa*) extract shows potential as a low-cost and efficient milkclotting agent. International Dairy Journal, 32 46–52. https://doi.org/10.1016/j.idairyj.2013.03.001

Hannon, J. A., Kilcawley, K. N., Wilkinson, M. G., Delahunty, C. M., & Beresford, T. P. (2006). Production of Ingredient-Type Cheddar Cheese with Accelerated Flavor Development by Addition of Enzyme-Modified Cheese Powder. Journal of Dairy Science, 89 3749–3762. https://doi.org/10.3168/jds.S0022- 0302(06)72416-X

Hashem, A. M. (2000). Purification and properties of a milk-clotting enzyme produced by *Penicillium oxalicum*. Bioresource Technology, 75 219–222. https://doi.org/10.1016/S0960-8524(00)00055-9

Horne, D. S., & Lucey, J. A. (2017). Rennet-Induced Coagulation of Milk. Cheese. In Paul L.H. McSweeney, Patrick F. Fox, Paul D. Cotter, David W. Everett (Eds.), Cheese (Fourth Edition) (pp.115–143). Academic Press. https://doi.org/10.1016/B978-0-12-417012-4.00005-3

Jacob, M., Jaros, D., & Rohm, H. (2011). Recent advances in milk clotting enzymes. International Journal of Dairy Technology, 64 14–33. https://doi.org/10.1111/j.1471-0307.2010.00633.x

Karel, M. (1990). Encapsulation and controlled release of food components. In G. Schwartzberg, M.A. Rao (Eds.), Biotechnology and food process engineering (pp. 277-294). Marcel Dekker, New York.

Khattab, A. R., Guirguis, H. A., Tawfik, S. M., & Farag, M. A. (2019). Cheese ripening: A review on modern technologies towards flavor enhancement, process acceleration and improved quality assessment. Trends in Food Science & Technology, 88 343-360. doi:10.1016/j.tifs.2019.03.009

Kelly, P. (2011). Milk Protein Products | Milk Protein Concentrate. In Fuquay JW, Fox PF and McSweeney PLH (Eds.), Encyclopedia of Dairy Sciences, Second Edition (pp. 848–854). Academic Press. https://doi.org/10.1016/B978-0-12-374407-4.00346-0

Kilcawley, K., Wilkinson, M., & Fox, P. (2000). A survey of the composition and proteolytic indices of commercial enzyme-modified Cheddar cheese. International Dairy Journal 10 181–190. https://doi:10.1016/s0958-6946(00)00029-7

Kilcawley, K. N., Wilkinson, M. G., & Fox, P. F. (2006). A novel two-stage process for the production of enzyme-modified cheese. Food Research International, 39 619–627. https://doi.org/10.1016/j.foodres.2005.12.006

Kilcawley, K. N., Nongonierma, A. B., Hannon, J. A., Doolan, I. A., & Wilkinson, M. G. (2012) Evaluation of commercial enzyme systems to accelerate Cheddar cheese ripening. International Dairy Journal, 26 50–57. https://doi.org/10.1016/j.idairyj.2012.03.015

Kleekayai, T., & FitzGerald, J. R. (2022). Manufacture of Milk and Whey Products: Protein Hydrolysates and Peptides. In Paul L.H. McSweeney, John P. McNamara (Eds.), Encyclopedia of Dairy Sciences (Third Edition) (pp. 154-166). Academic Press. https://doi.org/10.1016/B978-0-12-818766-1.00183-5

Koirala, S., Prathumpai, W., & Anal, A. K. (2021). Effect of ultrasonication pretreatment followed by enzymatic hydrolysis of caprine milk proteins and on antioxidant and angiotensin converting enzyme (ACE) inhibitory activity of peptides thus produced. International Dairy Journal, 118 105026. https://doi.org/10.1016/j.idairyj.2021.105026

Korhonen, H., & Pihlanto, A. (2006.) Bioactive peptides: Production and functionality. International Dairy Journal, 16 945–960. https://doi.org/10.1016/j.idairyj.2005.10.012

Korhonen, H. (2009). Milk-derived bioactive peptides: From science to applications. Journal of Functional Foods, 1 177–187. https://doi.org/10.1016/j.jff.2009.01.007

Kumar, A., Grover, S., Sharma, J., & Batish, V. K. (2010). Chymosin and other milk coagulants: sources and biotechnological interventions. Critical Reviews in Biotechnology, 30 243–258. https://doi.org/10.3109/07388551.2010.483459

Kumari, M., Sharma, A., Jagannadham, M. V. (2012). Religios in B, a milk-clotting serine protease from *Ficus religiosa*. Food Chemistry, 131 1295–1303. https://doi.org/10.1016/j.foodchem.2011.09.122

Lemes, A. C., Pavó, Y., Lazzaroni, S., Rozycki, S., Brandelli, A., & Kalil, S. J. (2016). A new milk-clotting enzyme produced by Bacillus sp. P45 applied in cream cheese development. LWT - Food Science and Technology, 66 217–224. https://doi.org/10.1016/j.lwt.2015.10.038

Leclerc, P. L., Gauthier, S. F., Bachelard, H., Santure, M., & Roy, D. (2002). Antihypertensive activity of casein-enriched milk fermented by *Lactobacillus helveticus*. International Dairy Journal, 12 995–1004. doi:10.1016/s0958-6946(02)00125-5

Mazorra-Manzano, M. A., Perea-Gutiérrez, T. C., Lugo-Sánchez, M. E., Ramirez-Suarez. J, C., Torres-Llanez, M. J., González-Córdova, A. F., & Vallejo-Cordoba, B. (2013). Comparison of the milk-clotting properties of three plant extracts. Food Chemistry 141 1902–1907. https://doi.org/10.1016/j.foodchem.2013.05.042

Mazorra-Manzano, M. A., Ramírez-Suarez, J. C., & Yada, R. Y. (2017). Plant proteases for bioactive peptides release: A review. Critical Reviews in Food Science and Nutrition, 1–17. doi:10.1080/10408398.2017.1308312

Mazorra-Manzano, M. A., Mora-Cortes, W. G., Leandro-Roldan, M. M., González-Velázquez, D. A., Torres-Llanez, M. J., Ramírez-Suarez, J. C., González- Córdova, F. A., & Vallejo-Córdoba, B. (2020). Production of whey protein hydrolysates with angiotensin-converting enzyme-inhibitory activity using three new sources of plant

proteases. Biocatalysis and Agricultural Biotechnology, 28 101724. https://doi.org/10.1016/j.bcab.2020.101724

Meng, F., Chen, R., Zhu, X., Lu, Y., Nie, T., Lu, F., & Lu, Z. (2018). Newly Effective Milk-Clotting Enzyme from *Bacillus subtilis* and Its Application in Cheese Making. Journal of Agricultural and Food Chemistry, 66 6162–6169. https://doi.org/10.1021/acs.jafc.8b01697

Merheb-Dini, C., Garcia, G. A. C., Penna, A. L. B., Gomes, E., & Silva, R. (2012). Use of a new milk-clotting protease from *Thermomucor indicae-seudaticae N31* as coagulant and changes during ripening of Prato cheese. Food Chemistry, 130 859–865. https://doi.org/10.1016/j.foodchem.2011.07.105

McDonald, J. K. (1985). An overview of protease specificity and catalytic mechanisms: aspects related to nomenclature and classification. The Histochemical Journal, 17 773–785. https://doi.org/10.1007/BF01003313

McSweeney, P. L. H., Sousa, M. J. (2000). Biochemical pathways for the production of flavour compounds in cheeses during ripening: a review. Lait, 80 293–324. https://doi.org/10.1051/lait:2000127

Mikhaylin, S., Boussetta, N., Vorobiev, E & Bazinet, L. (2017). High Voltage Electrical Treatments to Improve the Protein Susceptibility to Enzymatic Hydrolysis. ACS Sustainable Chemistry & Engineering, 5 11706–11714. https://doi.org/10.1021/acssuschemeng.7b03192

Mótyán, J., Tóth, F., Tőzsér, J. (2013). Research Applications of Proteolytic Enzymes in Molecular Biology. Biomolecules, 3 923–942. https://doi.org/10.3390/biom3040923

Moskowitz, G. J., & Noelck, S. S. (1987). Enzyme-Modified Cheese Technology. Journal of Dairy Science, 70 1761–1769. https://doi.org/10.3168/jds.S0022-0302(87)80208-4

Nasri, R., Abdelhedi, O., Nasri, M., & Jridi, M. (2022). Fermented protein hydrolysates: biological activities and applications. Current Opinion in Food Science, 43 120-127. https://doi.org/10.1016/j.cofs.2021.11.006.

Narwal, K. R., Bhushan, B., Pal, A., Panwar, A., & Malhotra, S. (2016). Purification, physico-chemico-kinetic characterization and thermal inactivation thermodynamics of milk clotting enzyme from *Bacillus subtilis* MTCC 10422. LWT - Food Science and Technology, 65 652–660. https://doi.org/10.1016/j.lwt.2015.08.065

Nielsen, D. S., Jakobsen, M. A. L, Geiker, R. W. N., & Bertram. C. H. (2022). Chemically acidified, live and heat-inactivated fermented dairy yoghurt show distinct bioactive peptides, free amino acids and small compounds profiles. Food Chemistry, 376 131919. https://doi.org/10.1016/j.foodchem.2021.131919.

Nomenclature Committee of the International Union of Biochemistry Molecular Biology. (1992). Enzyme Nomenclature 1992. Academic Press, Orlando

Nongonierma, A. B., & FitzGerald, R. J. (2011). Enzymes Exogenous to Milk in Dairy Technology | Proteinases. In Roginki, H., Fuquay, J. W. and Fox, P. F. (Eds)., Encyclopedia of Dairy Sciences (pp.289–296). Academic Press.

Oldfield, H. D. & Singh. (2005). Singh Functional properties of milk powders. C. Onwulata (Ed.), Encapsulated and powdered foods (pp.365-386), Taylor & Francis.

Park. S. Y., Gibbs, B. F., Lee, B.H. (1995). Effects of crude enzyme of *Lactobacilfus casei* LLG on water-soluble peptides of enzyme-modified cheese. Food Resarch International, 28,43-49.

Research and Markets Proteases Report - Global Market Trajectory & Analytics. (2021).https://www.researchandmarkets.com/reports/4805440/proteasesglobalmarket- trajectory-and-analytics#relb2-4987389

Ryhänen, E-L., Pihlanto-Leppälä, A., & Pahkala, E. (2001). A new type of ripened, low-fat cheese with bioactive properties. International Dairy Journal, 11 441– 447. https://doi.org/10.1016/S0958-6946(01)00079-6

Sakr, M., & Liu, S. (2014). A comprehensive review on applications of ohmic heating (OH). Renewable and Sustainable Energy Reviews, 39 262–269. https://doi.org/10.1016/j.rser.2014.07.061

Salum, P., Berktas, S., Cam, M., & Erbay, Z. (2022). Enzyme-modified cheese powder production: Influence of spray drying conditions on the physical properties, free fatty

acid content and volatile compounds. International Dairy Journal, 125 105241. https://doi.org/10.1016/j.idairyj.2021.105241

Sanchez, S., & Demain, A. L. (2017). Useful Microbial Enzymes-An Introduction. In Goutam Brahmachari (Ed.)., Biotechnology of Microbial Enzymes (pp.1-11). Academic Press. https://doi.org/10.1016/B978-0-12-803725-6.00001-7.

Sawant, R., & Nagendran, S. (2014). Protease: an enzyme with multiple Industrial applications. World journal of pharmacy and Pharmaceutical sciences, 3 568- 579.

Shivanna, S. K., & Nataraj, B. H. (2020). Revisiting Therapeutic and Toxicological Fingerprints of Milk-Derived Bioactive Peptides: An Overview. Food Bioscience, 38 100771. https://doi.org/10.1016/j.fbio.2020.100771

Shamtsyan, M., Dmitriyeva, T., Kolesnikov, B., & Denisova, N. (2014). Novel milk-clotting enzyme produced by *Coprinus lagopides* basidial mushroom. LWT Food Science and Technology, 58 343–347. https://doi.org/10.1016/j.lwt.2013.10.009

Shieh, C. J., Phan Thi, L. A., & Shih, I. L. (2009). Milk-clotting enzymes produced by culture of *Bacillus subtilis natto*. Biochemical Engineering Journal, 43 85– 91. https://doi.org/10.1016/j.bej.2008.09.003 Singh, H., & Ye, A. (2014). Interactions and Functionality of Milk Proteins in Food Emulsions. Milk Proteins, 359–386. https://doi.org/10.1016/B978-0-12- 815251-5.00012-8

Singh, R., Kumar, M., Mittal, A., Mehta, P.K. (2016). Microbial enzymes: industrial progress in 21st century. 3 Biotech, 6 174. https://doi.org/10.1007/s13205-016-0485-8

Soda, E. M., & Awad, S. (2011). Cheese | Accelerated Cheese Ripening. In Paul L.H. McSweeney, John P. McNamara (Eds.), Encyclopedia of Dairy Sciences (pp.795–798). Academic Press.

Salehi, M., Aghamaali, M. R., Sajedi, R. H., Asghari, S. M., & Jorjani, E. (2017). Purification and characterization of a milk-clotting aspartic protease from *Withania coagulans* fruit. International Journal of Biological Macromolecules, 98 847–854. https://doi.org/10.1016/j.ijbiomac.2017.02.034

Shah, M. A., Mir, S. A., & Paray, M.A. (2014). Plant proteases as milk-clotting enzymes in cheesemaking: a review. Dairy Science & Technology, 94 5–16. https://doi.org/10.1007/s13594-013-0144-3

Skrzypczak, K., Gustaw, W., Fornal, E., Kononiuk, A., Michalak-Majewska, M., Radzki, W., & Wa´sko, A. (2020). Functional and Technological Potential of Whey Protein Isolate in Production of Milk Beverages Fermented by New Strains of *Lactobacillus helveticus*. Applied Sciences, 10 7089. https://doi.org/10.3390/app10207089

Spök, A. (2006). Safety Regulations of Food Enzymes. Food Technology and Biotechnology, 44 197–209.Tao, L. (2011). Contribution of exopeptidases to formation of nonprotein nitrogen during ensiling of alfalfa. Journal of Dairy Science, 94 3928–3935.https://doi.org/10.3168/jds.2010-3752

Tavano, O. L. (2013). Protein hydrolysis using proteases: An important tool for food biotechnology. Journal of Molecular Catalysis B: Enzymatic, 90 1–11. https://doi.org/10.1016/j.molcatb.2013.01.011.

Tavano, O. L. (2015). Proteases as a Tool in Food Biotechnology. Advances in Food Biotechnology, 207–220.

Tavano, O. L., Berenguer-Murcia, A., Secundo, F., & Fernandez-Lafuente, R. (2018). Biotechnological Applications of Proteases in Food Technology. Comprehensive Reviews in Food Science and Food Safety, 17 412–436. https://doi.org/10.1111/1541-4337.12326

Uluko, H., Zhang, S., Liu, L., Li, H., Cui, W., Xue, H., Zhaoa, L., Sunc, Y., Lua, J., & Lv, J. (2014). Pilot-scale membrane fractionation of ACE inhibitory and antioxidative peptides from ultrasound pretreated milk protein concentrate hydrolysates. Journal of Functional Foods 7 350–361.https://doi.org/10.1016/j.jff.2014.01.025

Uluko, H., Zhang, S., Liu, L., Tsakama, M., Lu, J., & Lv, J. (2015). Effects of thermal, microwave, and ultrasound pretreatments on antioxidative capacity of enzymatic milk protein concentrate hydrolysates. Journal of Functional Foods, 18 1138–1146. https://doi.org/10.1016/j.jff.2014.11.024

Vallejo, J. A., Ageitos, J. M., Poza, M., & Villa, T. G. (2012). Short communication: A comparative analysis of recombinant chymosins. Journal of Dairy Science, 95 609–613. https://doi.org/10.3168/jds.2011-4445

Visser, S. (1993) Proteolytic enzymes and their relation to cheese ripening and flavor: An overview. Journal of Dairy Science, 76 329-350. https://doi.org/10.3168/jds.S0022-0302(93)77354-3

Vranova, V., Rejsek, K., & Formanek, P. (2013). Proteolytic activity in soil: A review. Applied Soil Ecology 70 23–32. https://doi.org/10.1016/j.apsoil.2013.04.003

Xia, Y., Yuan, R., Weng, S., Wang, G., Xiong, Z., Zhang, H., & Ai, L. (2020). Proteolysis, lipolysis, texture and sensory properties of cheese ripened by *Monascus fumeus*. Food Research International, 137 109657. https://doi.org/10.1016/j.foodres.2020.109657

Zanutto-Elgui, M. R., Vieira, J. C. S., do Prado, D. Z., Buzalaf, M. A. R., de Magalhães Padilha, P., & Fleuri, L. F. (2019). Production of milk peptides with antimicrobial and antioxidant properties through fungal proteases. Food Chemistry, 278 823-831. https://doi.org/10.1016/j.foodchem.2018.11.119

Zikiou, A., & Zidoune, M. N. (2018). Enzymatic extract from flowers of *Algerian spontaneous Cynara cardunculus* : Milk-clotting properties and use in the manufacture of a Camembert-type cheese. International Journal of Dairy Technology 71. https://doi.org/10.1111/1471-0307.12563

Yang, X., Zhang, Z., Zhang, W., Qiao, H., Wen, P., & Zhang, Y. (2022) Proteomic analysis, purification and characterization of a new milk-clotting protease from *Tenebrio molitor* larvae. Journal of Functional Foods, 89 104944. https://doi.org/10.1016/j.jff.2022.104944

Wehaidy, H. R., Abdel-Naby, M. A., Shousha, W. G., Elmallah, M. I. Y., & Shawky, M. M. (2018). Improving the catalytic, kinetic and thermodynamic properties of *Bacillus subtilis* KU710517 milk clotting enzyme via conjugation with polyethylene glycol. International Journal of Biological Macromolecules, 111 296–301. https://doi.org/10.1016/j.ijbiomac.2017.12.125

Wehaidy, H. R., Abdel, Wahab, W. A, Kholif, A. M. M., Elaaser, M., Bahgaat, W. K., & Abdel-Naby M, A. (2020). Statistical optimization of *B. subtilis* MK775302 milk clotting enzyme production using agro-industrial residues, enzyme characterization and application in cheese manufacture. Biocatalysis and Agricultural Biotechnology, 25 101589. https://doi.org/10.1016/j.bcab.2020.101589

Wilkinson, M. G., Doolan, I. A., & Kilcawley, K. N. (2011). Cheese | Enzyme-Modified Cheese. Encyclopedia of Dairy Sciences (pp.799–804). Academic Press.

3. CHAPTER 2: Manuscript prepared for submission to Biocatalysts and Agricultural Biotechnology.

Hydrolysis of whey protein and antioxidant activity of hydrolysates: Optimization by response surface methodology

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Abstract

Milk proteins are one of the most important sources of biological activity peptides. Medium and short peptides encrypted within the original protein sequence are released by the action of proteolytic enzymes. In this study, the hydrolysis of bovine whey protein isolate solutions (WPI) by commercial peptidases, was optimized using the response surface methodology (RSM). Degree of hydrolysis (DH) and antioxidant activity (ABTS and DPPH methodologies) were used as objective functions and hydrolysis time, temperature, and enzyme concentration as manipulated parameters. The model was statistically appropriate to describe the degree of hydrolysis (DH) and antioxidant activity from WPI solutions. RSM was effective in optimized hydrolysis conditions for the maximum DH and antioxidant activity values. In general, DH was significantly influenced by hydrolysis time, and antioxidant activity increased at higher temperatures (above 35 °C) and higher DH (%). This study indicates the potential use of commercial endopeptidases to produce protein hydrolysates with antioxidant activity.

Keywords: Commercial peptidase; Whey protein hydrolysate; RSM optimization; Bioactive peptide; Antioxidant activity.

Introduction

Several peptides with different biological activities, such as antioxidant, antihypertensive and antimicrobial, can be obtained through the enzymatic hydrolysis of milk proteins, caseins, and /or whey proteins (Brandelli et al., 2015; Paiva et al., 2022). These bioactive compounds are innovative alternatives for the food industry since possessing good properties, such as small molecule weight, low toxicity, strong free radical scavenging ability, and easy absorption (Song et al., 2020; Millan et al., 2022).

Specifically, bioactive antioxidant peptides have attracted a great deal of interest because of their safety and wide distribution properties, also the possibility to replace synthetic antioxidants (Zhang et al., 2009; Vastag et al., 2010; de Castro & Sato, 2014; Koirala et al., 2021;). Their antioxidant characteristics come from the ability to inactivate reactive oxygen species (ROS), scavenge free radicals, chelate pro-oxidative transition metals, and reduce hydroperoxides (de Castro & Sato, 2014). These mechanisms are based on their amino acid compositions and peptide sequences, which generally include 5–11 amino acids, such as proline, histidine, tyrosine, and tryptophan (Korhonen and Pihlanto, 2006; Zhou et al., 2012).

Commercial peptidases have been successfully tested for the production of bioactive hydrolysates from milk, including whey proteins (Butré et al., 2012; de Castro & Sato, 2014; Agustina Eberhardt, 202; Du et al., 2022; Ha et al., 2022). Enzymes from microorganisms appear as attractive biocatalysts to obtain protein hydrolysates and bioactive peptides on a commercial scale. Peptidase from microorganism sources such as bacteria and fungi is mainly represented by *Bacillus* and *Aspergillus* genera (Gibbs et al., 2004; Aguirre et al., 2008; Singh et al., 2016), and has been successfully

tested to obtain bioactive hydrolysates from whey proteins (Corrêa et al., 2014; Eberhardt et al., 2021).

Intending hydrolysates with bioactivity properties, some factors must be considered, for instance, the influence of hydrolysis parameters, the type and enzyme concentration, substrate concentration, and also the time and temperature of hydrolysis (Vaštag et al., 2010). Response surface methodology (RSM) is a valuable statistical and mathematical set of tools for modeling and optimizing experiments. As the hydrolysis process depends on many parameters, RSM stands out in optimizing the hydrolysis conditions and determining the experimental effects' optimal settings (Quirós et al., 2012; Mansinhbhai et al., 2022). In the last few years, different studies have demonstrated the successful use of this technique at the maximum release of bioactive peptides from whey proteins (van der Ven et al., 2002; Guo et al., 2009; Contreras et al., 2011; Tavares et al., 2011; Quirós et al., 2012; Naik et al., 2013).

Aimed the investigate new peptidases to release protein hydrolysates with antioxidant properties, in the present study, whey protein solutions were hydrolyzed with two commercial endopeptidases (Maxipro® PSP and Maxipro® TNP), from fungal and bacterial sources, respectively. The effects of process parameters: comprising time, the temperature of hydrolysis, and enzyme concentration on the degree of hydrolysis (DH) and antioxidant activity were studied using the Box–Behnken design (BBD).

Material and methods

Materials

Whey protein isolate (WPI, 92% w/w protein) was obtained from Arla Foods Ingredients (Arla Oy, Arla Foods UK). 1-diphenyl-2-pycryl hydrazyl (DPPH) and 2,2-azino-bis (3-ethylben- zothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

The endopeptidases (Maxipro® PSP and Maxipro® TNP) were acquired from DSM (Heerlen, Netherlands). Maxipro® PSP (Enzyme 1) cleaves specifically at the carboxy side of the proline of proteins, considering a fungal acid peptidase from a selected strain of *Aspergillus niger,* which means that its optimum pH is at acid conditions (3 to 4), with the optimal temperature at 55°C. Maxipro® TNP (Enzyme 2), possesses broad peptide bond specificity and the ability to efficiently hydrolyze proteins, it is a bacterial neutral peptidase from a selected strain of *Bacillus amyloliquefaciens*, a neutral-protease that presents an optimum pH at neutral conditions, near pH 7, and works at its optimal temperature among 40-50°C.

Methods

Enzymatic hydrolysis of WPI

WPI was dispersed in acetate buffer (50 mM, pH 4,0) or phosphate buffer (50 mM, pH 7,0) to obtain 6% w/w protein in the dispersion solution. Then, the solutions were stirred for 2 h at room temperature and kept overnight at 4°C, prior to the enzymatic hydrolysis. The hydrolysis was carried out according to Alizadeh and Aliakbarlu (2020), with some modifications. WPI solutions were placed in an incubation shaker set at 100 rpm. Enzyme 1 and Enzyme 2 were added to WPI dispersed in

acetate buffer and phosphate buffer, respectively, considering their optimal pH, ensuring a final concentration of 1%, 3%, and 5% w/w in the protein dry matter. The enzymatic hydrolysis was performed at 20, 35, and 50 °C for 1, 9, and 17h. The reaction was stopped by adding 1 mol/L NaOH to reach pH 11. Then, the reaction solutions were centrifuged at 3260×g for 10 min at 4 °C, aiming to separate the hydrolyzed and non-hydrolyzed protein chains. The supernatant was stored at -80 °C for further analysis.

Degree of hydrolysis (DH)

The DH of the hydrolyzed solutions was performed according to the method described by Alizadeh and Aliakbarlu (2020), with some modifications. The absorbance of the hydrolyzed solutions was measured in a spectrophotometer at 280nm, using quartz cuvettes. First, the absorbance of the total peptides was measured, then the trichloroacetic acid solution (200 g/L) was mixed with an equal volume of the different supernatants, these solutions were kept overnight at 8°C. Subsequently, the samples were centrifugated (3260×g for 10 min at 4°C) to obtain the absorbance measurement of the soluble peptides. The DH process was calculated according to Eq. (1):

$$DH\ (\%) = \frac{A_{280}\ TCA\ 10\%\ Soluble\ peptides\ in\ the\ supernatant}{A_{280}\ Total\ peptides\ in\ the\ supernatant} \cdot 100 \qquad (1)$$

Where A_{280} is the absorbance at 280 nm, which was measured using a spectrophotometer in the supernatant before (total peptides) and after (soluble peptides) precipitation with 10% TCA.

Antioxidant activity assays

ABTS+ radical scavenging activity

ABTS+ radical scavenging activity was determined according to the method described by Liu et al. (2019), with some modifications. The working solution was prepared by mixing two stock solutions of 7.00 mmol/L ABTS and 2.45 mmol/L potassium persulfate in equal amounts. This mixture was incubated in the dark for 12–16 h at room temperature. 5 µL of each hydrolyzed sample was mixed with 5 mL of ABTS+ solution (working solution diluted in ethanol 80% w/w, until an absorbance of 0.70 ± 0.02 at 734 nm was reached). The mixture was incubated in the dark for 6 min, and the absorbance was determined at 734 nm using a microplate reader (Multiskan GO Microplate Spectrophotometer, Thermo Fisher Scientific Oy, Vantaa, Finland). The ABTS+ radical scavenging activity was calculated by Eq. (2):

$$ABTS^{+} radical\ scavening\ activity\ (\%) = (Ac - As)\ \cdot \frac{100}{Ac} \quad (2)$$

Where Ac is the absorbance of the control (5 mL ABTS+ solution alone with 5 µL Milli-Q water with adjusted pH) and As is the absorbance of the hydrolysates samples (5 mL ABTS+ solution with 5 µL hydrolysate sample).

DPPH radical scavenging activity

DPPH radical scavenging activity was determined according to the method described by Liu et al. (2019), with some modifications. 200 µL of each hydrolyzed sample was added to 200 µL of 0.2 mM DPPH in ethanol 80%. The solution was then mixed vigorously and stored for 15 min at room temperature in the dark. The mixture was centrifuged for 10 min at 3260×g at 4 °C and the absorbance of the supernatant

was measured at 517 nm using a microplate reader. The DPPH radical scavenging activity was calculated using Eq. (3):

$$DPPH\ radical\ scavening\ activity\ (\%) = 100 - \frac{As-Ac}{Ab} \cdot 100 \quad (3)$$

Where As is the absorbance of the hydrolysate sample added to DPPH, Ac is the absorbance of the control (ethanol was added instead of DPPH), and Ab is the absorbance of the blank (Milli-Q water with adjusted pH instead of the sample).

Experimental design, and statistical analysis

The effects of three processing factors: temperature, enzyme concentration, and reaction time, were studied on DH and antioxidant activities of the corresponding hydrolysates. The response surface methodology (RSM) using Box–Behnken design (BBD) was selected for designing, modeling, and optimization of the process parameters. The design generation was performed using the R programming language version 4.0.2 (R Core Team, 2020) and RStudio v.1.3.959 (RStudio Team, 2020), and the RSM package (Lenth, 2009). The real values of independent variables and their levels are shown in Table 1. The experimental design consisted of 15 runs with three replicates at the central point, as shown in Table 2. The experimental sequence was randomized to minimize the effects of the uncontrolled factors. A quadratic polynomial regression model, as shown in Eq. (4), was used to analyze the data obtained.

$$\hat{Y} = \beta + \sum \beta_i X_i + \sum \beta_{ii} X_{ii}^2 + \sum \beta_{ij} X_i X_j \quad (4)$$

Where $\hat{Y}$ represents the response variable, β_0 is the constant term, βi is the linear coefficient, βii is the quadratic coefficient, and βij is the interaction coefficient.

Statistical analyses were performed using RSM package in R programming language. The Akaike information criterion (AIC) was applied to automatically select the best model. The variability and accuracy of the developed model were determined by the coefficient of determination (R^2), adjusted R^2. The optimization was performed by using a constrained nonlinear optimization method, based on Sequential Quadratic Programming (SQP) algorithm, conducted using the R package NlcOptim (Chen & Yin, 2019). The surface plots were generated using the default methods in base R: the "Persp", and "Plot" functions (Becker, Chambers, & Wilks, 1988). The Response surface plots (3D) were made keeping one independent variable at the central level and changing the other two. The individual effects plots and interaction plots were generated using the R package ggplot2 (Wickham, 2016).

Results and discussion

Model performance appraisal on the regression elements

It is well known that temperature (A), hydrolysis time (B), and enzyme concentration (C) affect the enzymatic hydrolysis and antioxidant activity of whey protein hydrolysates. RSM approach was applied to investigate the influence of these variables as well as to optimize the hydrolysis conditions of WPI solutions to obtain the most powerful antioxidant hydrolysate from whey proteins.

The results of the t-test for the regression coefficients on DH and antioxidant activity for the significance of the polynomial model are presented in Table 3.

Some statistics are crucial to measuring the model adequacy, including the coefficient of determination (R^2) and the adjusted coefficient of determination (adjusted-R^2). These parameters are presented in Table 3. As seen in Table 3, the adjusted R^2 values vary from 0.7215 to 0.9768, indicating that the models are adapted to the responses,

and suitable to predict DH and the antioxidant activity of the hydrolyzed samples. Figure 1 shows the goodness of fit between experimental and predicted values. The data points fall close to the regression line (Figure 1), mainly for antioxidant response (ABTS assay) which achieved higher adjusted-R^2 values of 0.9884 and 0.9472 (Table 3).

Effects of temperature, hydrolysis time, and enzyme concentration on different responses

Degree of hydrolysis (DH)

DH (%) measures the content of peptides and bonds cleaved in the substrate by a peptidase action, higher DH values indicate a greater release of amino groups (Corrêa et al., 2011). Based on the RSM approach, the dependence of DH on process parameters can be confirmed through Table 3 and Table 4.

Regarding Enzyme 1 it is observed that the linear effects A, B, and C were statistically significant (Table 3), considering the maximum level of significance adopted $\alpha = 0.05$, with the reaction time presented as the most significant effect ($p < 0.001$). These significant effects (A, B, and C) presented a positive influence on DH response, confirmed by the positive signal view in the second-order polynomial equations (Table 4), also illustrated in Figure 2 (a), where it can be noticed that the change in the levels of A, B, and C produces a positive effect, i.e., it increases the response DH. Concerning the quadratic effects of the control variables, it can be seen in Table 3 that none showed statistical significance.

In the interaction terms, B x C presented a positive effect (Table 4), considered a statistical significance, p-value < α (Table 3). Figure 3 (a) showed the interaction plots for DH. According to Figure 3 (a), the first panel from left to right, the greater the

reaction time (B), the greater the positive effect of the temperature (A), and, considering the third panel, the greater the enzyme level (C), greater is the positive effect of the reaction time (B). Moreover, the response surface plot (3D), seen in Figure 4 (a), showed that in the interaction's effects between time and enzyme concentration and time and temperature, the reaction time had a greater influence on DH results.

As seen in Table 2, the highest DH value of 1,19% was achieved at the maximum hydrolysis time (17h, 35 °C, 5% enzyme concentration). Other authors also related time as a significant parameter to increasing DH (%) (Corrêa et al., 2011; Kheroufi et al., 2022). However, there is no guarantee that much hydrolysis time will increase DH, since this will be directly influenced by enzyme specificity, and enzymes could not further hydrolyze the remaining bonds within the generated peptides (Corrêa et al., 2011).

For Enzyme 2, DH was significantly influenced ($p < \alpha$) by the linear effects A, B, and C (Table 3). As viewed in the second-order polynomial equation (Table 4), both effects (A, B, and C) presented a positive influence on DH response. The individual effects plot in Figure 5 (a) noticed these positive effects on DH, where the change in the levels of A, B, and C improved DH, among them the raised in temperature (A) presented the highest influence on DH increase. In the quadratic effects, it can be seen that only A^2 showed statistical significance, p-value > α (Table 3).

Regarding interaction terms, A x C showed a significant positive effect on DH (Table 3). Figure 6 (2) illustrates the influence of those interaction effects (A x B, A x C, and B x C). Considering the significant interaction, the positive effect of the temperature (A) is highest when combined with the highest level of Enzyme (C). In the response surface plots (3D) (Figure 7 (a)), it is observed that the interaction between temperature and hydrolysis time had a positive influence on DH after 10h, above 40

°C, also enzyme concentration increased the response in its higher levels (above 4%). For enzyme 2 the highest DH of 5,16% occurred keeping temperature and enzyme concentration in their maximum conditions (50°C, 9h hydrolysis time, 5% of enzyme concentration).

According to these results, Enzyme 2, a neutral endopeptidase, achieved the highest DH (5.16%) on WPI hydrolysates. Protein hydrolysis by peptidase action is dependent on enzyme specificity, and also substrate type, which influences hydrolysis reaction, such as the disposition of the cleavage regions in the protein structure (Kheroufi et al., 2022). Regarding the peptidase type, the hydrolysis of a pH-neutral peptidase proceeded at an intermediate rate, whereas acid peptidase occurs slower, this may be attributed to the enzyme-substrate specificity, reaction environment, and substrate limitation (Koirala et al., 2021). These results are similar to the investigations of Eberhardt et al. (2021) that highlighted a commercial enzyme from *Bacillus licheniformis* presented a greater DH (%) than a peptidase from *Aspergillus oryzae*, explained by the different catalytic actions among them, which reflects on their specific accessibility in the whey protein chain.

From Table 2, DH between 0.33-5.16% were recorded for hydrolysis under different combinations of temperature, hydrolysis time, and enzyme concentration. Similar to the results of Tavares et al (2011) the hydrolysis degree values obtained in this study weren't high (Table 2). This may be due to the specificity of the enzymes used and the influence of the conditions studied, for example, hydrolysis conditions (enzyme-to-substrate ratio, temperature, pH, and time), and the source of the protein (Chaturika et al., 2014). Native whey proteins are not easily hydrolyzed by enzymes, due to their compact tertiary structure that hides most of the active substrate (Wijayanti et al., 2019). Prior enzymatic hydrolysis treatments can be used to enable peptidases'

action on whey proteins, such as physical and/or chemical denaturation (Uluko et al., 2014; Wijayanti et al., 2019; Alizadeh & Aliakbarlu 2020).

Antioxidant activity – ABTS and DPPH assays

Unlike typical works which perform only a single antioxidant assay in RSM studies (Hussein et al., 2020), current work reported two antioxidant activities, i.e., DPPH radical scavenging and ABTS+ radical scavenging activities. These methods are based on the antioxidant reaction with an organic radical 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl (DPPH) (Mareček et al., 2017).

After hydrolysis with enzymes 1 and 2, ABTS and DPPH radical scavenging activity showed values ranging from 1.30 to 13.17% and from 1.00 to 12.12%, respectively (Table 2).

The statistical influence of the studied effects on antioxidant activity by ABTS assay and DPPH assay are described in Table 3 and Table 4. Regarding enzyme 1, the results presented in Table 3 showed that all linear terms (A, B, and, C) had a significant influence on ABTS antioxidant activity, able to increase this response, due to a positive effect, as viewed in the equation (Table 4). In Figure 2 (b) it is observed that the higher the levels of A, B, and C, the greater the ABTS antioxidant activity, mainly due to the increase in temperature, which had the most significant positive influence. For quadratic terms, A^2 and B^2 were significant, presenting positive and negative influences on the response, with convexity upward and downward, respectively (Table 3 and Table 4). For interaction terms, only B x C was significant (Table 3), presenting a positive effect (Table 4). Main effects are graphically dominant when compared to quadratic effects, as can be attested by the magnitude and

significance of the effects. As illustrated in Figure 3 (b) and response surface plots (3D) (Figure 4 (b)), temperature showed the most relevant influence on ABTS antioxidant activity response.

On the antioxidant activity of DPPH, the linear effects A and C had a positive and significant influence on the response results (Table 3, Table 4). The graph of individual effects (Figure 2 (c)) showed that the reaction time had the lowest linear positive effect on DPPH antioxidant activity. Regarding the quadratic terms, only A^2 had a significant and positive effect, therefore with convexity upward, on the antioxidant activity of DPPH (Table 3, Table 4). Figure 3 (c) and Figure 4 (c) illustrate the interaction between the independent variables and their interactive effects on the response. As seen, the increase in temperature (above 35 °C) and the concentration of enzymes at the highest level (5%) are the major factors that influenced the increased antioxidant activity (DPPH assay).

For enzyme 2, all linear terms (A, B, and C) had a positive and significant influence (Table 3, Table 4) on ABTS antioxidant activity. An increase in ABTS antioxidant activity is observed when A, B, and C are at their highest levels (Figure 5 (b)), this was observed mainly in temperature (from 30°C) and enzyme concentration (from 2%). Regarding quadratic terms, only A^2 was significant, showing a positive influence on ABTS antioxidant activity response (Table 3 and Table 4). The interactive terms, A x C, and B x C were significant (Table 3). These interactions are illustrated in Figure 6 (b) and Figure 7 (b), it is observed that the maximum enzyme concentration and temperature (above 40 °C) were the main effects in increasing ABTS antioxidant activity.

Similar to enzyme 1, in DPPH antioxidant activity response, the linear terms A and C presented significant and positive effects (Table 3, Table 4). Figure 5 (c) showed

temperature as the major influence in increasing DPPH antioxidant activity, while the reaction time had a small contribution. In quadratic terms, only A^2 was significant, presenting a positive influence on this response (Table 3, Table 4). Regarding interaction terms, it is confirmed that temperature had the greatest influence on DPPH antioxidant activity, while reaction time had no influence, and enzyme concentration improved DPPH antioxidant activity only at its highest level (5%) (Figure 6 (c)). These effects were seen in response surface plots (3D) (Figure 7 (c)), in which the increase in DPPH antioxidant activity occurred at temperatures above 35 °C, while time and enzyme concentration presented less influence on this response.

The different results of antioxidant activity observed in Table 2 could be attributed to the specificity of each enzyme on the protein substrate, and also the release of peptides with different molecular masses, amino acid sequences, and antioxidant activities (de Castro and Sato 2014; Eberhardt et al. 2021). In general, the results showed that temperature above 35/40°C, was the major factor that contributed to antioxidant activity increase (ABTS and DPPH assays). This effect is explained by higher temperatures (close to 50 °C) influencing the unfolding of protein molecules and the consequent release of minor hydrophobic groups (Vaštag et al., 2010).

Furthermore, for both enzymes, the higher antioxidant activity values (ABTS and DPPH assays) were achieved when DH is increased (Table 2). The antioxidant activities are usually related to the release of peptides with low molecular mass, an increase in the number of ionizable groups, and exposure of hidden hydrophobic groups resulting from protein hydrolysis (Sarmadi & Ismail, 2010; Adjonu et al. 2013; de Castro and Sato 2014; Eberhardt et al. 2021). As seen in Table 2, the highest antioxidant activity of Enzyme 2, 13.17%, is correlated with the highest DH (%), considering the same peptidase.

Therefore, antioxidant activity results, following both assays, ABTS and DPPH, suggest that low molecular weight peptides were the main responsible for increased antioxidant activity response. (Athira et al., 2015; Eberhardt et al. 2021). These results suggested that hydrolysates obtained from higher DH (%) may prevent free radical damage in biological systems since these small hydrolysates can act as antioxidant compounds. Whey protein hydrolysates can be used in the food industry as a bio-antioxidant ingredient to avoid deteriorative processes such as lipid oxidation, causing unacceptable taste or texture, color, loss of nutritive value, and shorter shelf life (Dryáková et al. 2010; Eberhardt et al. 2019).

Optimized Hydrolysis Condition - Attaining optimum condition

From the experimental stationary points, it wasn't possible to achieve the parameters aimed at a maximum response of DH and antioxidant activity, because these points are outside the experimental region, or in some cases, only a minimal point was found due to the convexity of the response surfaces (Figure 4, Figure 7). Thus, in order to obtain the maximum values from DH and antioxidant activity (ABTS and DPPH) responses, the optimization of the model was performed using nonlinearly constrained optimization, through the sequential quadratic programming (SQP) algorithm in which the maximum values of DH and antioxidant activity were set as the goal (Joyce & Leung, 2013). Then, regarding the conditions applied in this work, the results of maximum DH and antioxidant activity were seen in Table 5.

Since SQP algorithm seeks the maximum constrained value of the responses, the temperature level was set to the maximum (50 º C), except for the response DH on enzyme 1 (43.23 º C). Reaction time presented optimum levels from 10.95 to 16.51 h, in the responses of enzyme 1, to optimize antioxidant activity and DH. For enzyme

2 the variation in Time was lowest (11.02 to 12.11 h). Finally, the Enzyme content was set nearby the maximum level considered in the experiments varying from 4.81 to 4.94 %.

Conclusions

This study represents a pioneer work on the optimization of whey protein concentrate hydrolysis using Maxipro® PSP and Maxipro® TNP to generate biopeptides with antioxidant activities. Response surface modeling was shown to be an effective method to optimize several parameters of the hydrolysis process, resulting in hydrolysates with maximum DH and antioxidant activity, considering the conditions studied. The experimental values agreed with the predicted value, suggesting a good fit between the models and the experimental data.

In general, the increasing DH leads to higher antioxidant activities for both hydrolysates, using Maxipro® PSP and Maxipro® TNP. However, WPI hydrolyzates obtained from Maxipro® TNP action presented the highest values for DH (5.16%) and antioxidant activity (13.7% ABTS and 12.12% DPPH assay), achieved at a temperature of 50 °C, a reaction time of 9 h, and 5% enzyme concentration. Therefore, these hydrolysates have been shown to have a certain antioxidant potential and may be promising natural antioxidant additives. However, more research is needed to isolate the individual peptides responsible for the antioxidant activity of WPI hydrolysates and identify their amino acid sequences.

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References

Adjonu R, Doran G, Torley P and Agboola S (2013) Screening of whey protein isolate hydrolysates for their dual functionality: Influence of heat pre-treatment and enzyme specificity. Food Chemistry 136 1435–1443.

Aguirre, L., Garro, M. S., & Savoy de Giori, G. (2008). Enzymatic hydrolysis of soybean protein using lactic acid bacteria. Food Chemistry, 111(4), 976–982. https://doi.org/10.1016/j.foodchem.2008.05.018

Alizadeh, O., & Aliakbarlu, J. (2020). Effects of ultrasound and ohmic heating pretreatments on hydrolysis, antioxidant and antibacterial activities of whey protein concentrate and its fractions. LWT, 131. https://doi.org/10.1016/j.lwt.2020.109913

Athira, S., Mann, B., Saini, P., Sharma, R., Kumar, R., & Singh, A. K. (2015). Production and characterisation of whey protein hydrolysate having antioxidant activity from cheese whey. Journal of the Science of Food and Agriculture, 95(14), 2908–2915. https://doi.org/10.1002/jsfa.7032

Becker, R. A., Chambers, J. M., & Wilks, A. R. (1988). The New S Language. Wadsworth & Brooks/Cole. Pacific Grove, CA.

Brandelli, A., Daroit, D. J., & Corrêa, A. P. F. (2015). Whey as a source of peptides with remarkable biological activities. Food Research International, 73, 149–161. https://doi.org/10.1016/j.foodres.2015.01.016

Butré, C. I., Wierenga, P. A., & Gruppen, H. (2012). Effects of ionic strength on the enzymatic hydrolysis of diluted and concentrated whey protein isolate. Journal of Agricultural and Food Chemistry, 60(22), 5644–5651. https://doi.org/10.1021/jf301409n

Chaturika Jeewanthi, R. K., Lee, K., & Paik, D. (2014). Improved Functional Characteristics of Whey Protein Hydrolysates in Food Industry. Korean Journal for Food Science of Animal Resources, 35(3), 350-359. https://doi.org/10.5851/kosfa.2015.35.3.350

Chen, X. & Yin. X. (2019). NlcOptim: Solve nonlinear optimization with nonlinear constraints. R Package Version 0.6. https://CRAN.R-project.org/package=NlcOptim

Contreras, M. del M., Hernández-Ledesma, B., Amigo, L., Martín-Álvarez, P. J., & Recio, I. (2011). Production of antioxidant hydrolyzates from a whey protein concentrate with thermolysin: Optimization by response surface methodology. LWT - Food Science and Technology, 44(1), 9–15. https://doi.org/10.1016/j.lwt.2010.06.017

Corrêa, A. P. F., Daroit, D. J., Coelho, J., Meira, S. M., Lopes, F. C., Segalin, J., Risso, P. H., & Brandelli, A. (2011). Antioxidant, antihypertensive and antimicrobial properties of ovine milk caseinate hydrolyzed with a microbial protease. Journal of the Science of Food and Agriculture, 91(12), 2247–2254. https://doi.org/10.1002/jsfa.4446

Corrêa, A. P. F., Daroit, D. J., Fontoura, R., Meira, S. M. M., Segalin, J., & Brandelli, A. (2014). Hydrolysates of sheep cheese whey as a source of bioactive peptides with antioxidant and angiotensin-converting enzyme inhibitory activities. Peptides, 61, 48–55. https://doi.org/10.1016/j.peptides.2014.09.001

Cui, Q., Sun, Y., Cheng, J., & Guo, M. (2022). Effect of two-step enzymatic hydrolysis on the antioxidant properties and proteomics of hydrolysates of milk protein concentrate. Food Chemistry, 366. https://doi.org/10.1016/j.foodchem.2021.130711

de Castro, R. J. S., & Sato, H. H. (2014). Advantages of an acid protease from Aspergillus oryzae over commercial preparations for production of whey protein hydrolysates with antioxidant activities. Biocatalysis and Agricultural Biotechnology, 3(3), 58–65. https://doi.org/10.1016/j.bcab.2013.11.012

de Oliveira, L. G., de Paiva, A. P., Balestrassi, P. P., Ferreira, J. R., da Costa, S. C., & da Silva Campos, P. H. (2019). Response surface methodology for advanced manufacturing technology optimization: theoretical fundamentals, practical guidelines, and survey literature review. International Journal of Advanced Manufacturing Technology, 104(5–8), 1785–1837. https://doi.org/10.1007/s00170-019-03809-9

Du, X., Jing, H., Wang, L., Huang, X., Wang, X., & Wang, H. (2022). Characterization of structure, physicochemical properties, and hypoglycemic activity of goat milk whey protein hydrolysate processed with different proteases. LWT, 159. https://doi.org/10.1016/j.lwt.2022.113257

Dryáková, A., Pihlanto, A., Marnila, P., Čurda, L., & Korhonen, H. J. T. (2010). Antioxidant properties of whey protein hydrolysates as measured by three methods. European Food Research and Technology, 230(6), 865–874. doi:10.1007/s00217-010-1231-9

Eberhardt, A., López, E. C., Ceruti, R. J., Marino, F., Mammarella, E. J., Manzo, R. M., & Sihufe, G. A. (2019). Influence of the degree of hydrolysis on the bioactive properties of whey protein hydrolysates using Alcalase®. International Journal of Dairy Technology. doi:10.1111/1471-0307.12606

Eberhardt, A., López, E. C., Marino, F., Mammarella, E. J., Manzo, R. M., & Sihufe, G. A. (2021). Whey protein hydrolysis with microbial proteases: Determination of kinetic parameters and bioactive properties for different reaction conditions. International Journal of Dairy Technology, 3, 489–504. https://doi.org/10.1111/1471-0307.12795

Foegeding, E., Davis, J. P., Doucet, D., & McGuffey, M. K. (2002). Advances in modifying and understanding whey protein functionality. Trends in Food Science & Technology, 13(5), 151-159. https://doi.org/10.1016/S0924-2244(02)00111-5

Gibbs, B. F., Zougman, A., Masse, R., & Mulligan, C. (2004). Production and characterization of bioactive peptides from soy hydrolysate and soy-fermented food. Food Research International, 37(2), 123–131. https://doi.org/10.1016/j.foodres.2003.09.010

Guo, Y., Pan, D., & Tanokura, M. (2009). Optimisation of hydrolysis conditions for the production of the angiotensin-I converting enzyme (ACE) inhibitory peptides from whey protein using response surface methodology. Food Chemistry, 114(1), 328–333. https://doi.org/10.1016/j.foodchem.2008.09.041

Ha, M., El-Din Bekhit, A., McConnell, M., & Carne, A. (2022). A simple method for enrichment of β-lactoglobulin from bovine milk whey involving selective hydrolysis by two fungal protease preparations. Food Chemistry, 368. https://doi.org/10.1016/j.foodchem.2021.130820

Hussein, F. A., Chay, S. Y., Zarei, M., Auwal, S. M., Hamid, A. A., Wan Ibadullah, W. Z., & Saari, N. (2020). Whey protein concentrate as a novel source of bifunctional peptides with angiotensin-i converting enzyme inhibitory and antioxidant properties: RSM study. Foods, 9(1). https://doi.org/10.3390/foods9010064

Joyce, A. P., & Leung, S. S. (2013). Use of response surface methods and path of steepest ascent to optimize ligand-binding assay sensitivity. Journal of Immunological Methods, 392(1–2), 12–23. https://doi.org/10.1016/j.jim.2013.02.019

Kananen, A., Savolainen, J., Mäkinen, J., Perttilä, U., Myllykoski, L., & Pihlanto-Leppälä, A. (2000). Influence of chemical modification of whey protein conformation on hydrolysis with pepsin and trypsin. International Dairy Journal, 10(10), 691-697. https://doi.org/10.1016/S0958-6946(00)00094-7

Koirala, S., Prathumpai, W., & Anal, A. K. (2021). Effect of ultrasonication pretreatment followed by enzymatic hydrolysis of caprine milk proteins and on antioxidant and angiotensin converting enzyme (ACE) inhibitory activity of peptides thus produced. International Dairy Journal, 118. https://doi.org/10.1016/j.idairyj.2021.105026

Lee, D. H., Kim, S. H., & Byun, J. H. (2020). A method of steepest ascent for multiresponse surface optimization using a desirability function method. Quality and Reliability Engineering International, 36(6), 1931–1948. https://doi.org/10.1002/qre.2666

Lenth, R. V. (2009). Response-surface methods in R, using RSM. Journal of Statistical Software, *32*(7), 1–17. https://doi.org/10.18637/jss.v032.i07

Liu, L., Li, X., Du, L., Zhang, X., Yang, W., & Zhang, H. (2019). Effect of ultrasound assisted heating on structure and antioxidant activity of whey protein peptide grafted with galactose. LWT, 109, 130–136. https://doi.org/10.1016/j.lwt.2019.04.015

Mansinhbhai, C. H., Sakure, A., Maurya, R., Bishnoi, M., Kondepudi, K. K., Das, S., & Hati, S. (2022). Significance of whey protein hydrolysate on anti-oxidative, ACE-inhibitory and anti-inflammatory activities and release of peptides with biofunctionality: an in vitro and in silico approach. Journal of Food Science and Technology, 59(7), 2629–2642. https://doi.org/10.1007/s13197-021-05282-3

Millan, G. C. L., Veras, F. F., Stincone, P., Pailliè-Jiménez, M. E., & Brandelli, A. (2022). Biological activities of whey protein hydrolysate produced by protease from the Antarctic bacterium Lysobacter sp. A03. Biocatalysis and Agricultural Biotechnology, 43, 102415. https://doi.org/10.1016/j.bcab.2022.102415

Naik, L., Mann, B., Bajaj, R., Sangwan, R. B., & Sharma, R. (2013). Process optimization for the production of bio-functional whey protein hydrolysates: Adopting response surface methodology. International Journal of Peptide Research and Therapeutics, 19(3), 231–237. https://doi.org/10.1007/s10989-012-9340-x

Paiva, V. N., Martins, E., Machado, S. G., & Carvalho, A. F. de. (2022). Peptidases used in dairy technology: Current knowledge and relevant applications. Research, Society and Development, 11(7), e57211730367. https://doi.org/10.33448/rsd-v11i7.30367

Quirós, A., Hernández-Ledesma, B., Ramos, M., Martín-álvarez, P. J., & Recio, I. (2012). Short communication: Production of antihypertensive peptide HLPLP by enzymatic hydrolysis: Optimization by response surface methodology. Journal of Dairy Science, 95(8), 4280–4285. https://doi.org/10.3168/jds.2012-5459

R Core Team, 2020. R: A Language and Environment for Statistical Computing.

Rdsa, L. D. L. da, Santana, M. C., Avezedd, T. L., Brígoda, A. O. S., Gdddy, R., Pachecd, S., Mellonger-Solva, C., & Cabral, L. M. C. (2018). A comparison of dual-functional whey hydrolysates by the use of commercial proteases. Food Science and Technology (Brazil), 38, 31–36. https://doi.org/10.1590/fst.08417

RStudio Team, 2020. RStudio: Integrated Development Environment for R.

Ren, J., Zhao, M., Shi, J., Wang, J., Jiang, Y., Cui, C., Kakuda, Y., & Xue, S. J. (2008). Optimization of antioxidant peptide production from grass carp sarcoplasmic protein using response surface methodology. LWT - Food Science and Technology, 41(9), 1624–1632. https://doi.org/10.1016/j.lwt.2007.11.005

Sarmadi, B. H., & Ismail, A. (2010). Antioxidative peptides from food proteins: A review. In Peptides (Vol. 31, Issue 10, pp. 1949–1956). https://doi.org/10.1016/j.peptides.2010.06.020

Shahi, Z., Sayyed-Alangi, S. Z., & Najafian, L. (2020). Effects of enzyme type and process time on hydrolysis degree, electrophoresis bands and antioxidant properties

of hydrolyzed proteins derived from defatted Bunium persicum Bioss. press cake. Heliyon, 6(2), e03365. https://doi.org/10.1016/j.heliyon.2020.e03365

Song, R., Liang, T., Shen, Q., Liu, J., Lu, Y., Tang, C., Chen, X., Hou, T., & Chen, Y. (2020). The optimization of production and characterization of antioxidant peptides from protein hydrolysates of Agrocybe aegerita. LWT, 134. https://doi.org/10.1016/j.lwt.2020.109987

Spellman, D., O'Cuinn, G., & FitzGerald, R. (2009). Bitterness in Bacillus proteinase hydrolysates of whey proteins. Food Chemistry, 114(2), 440-446. https://doi.org/10.1016/j.foodchem.2008.09.067

Sun, Y., Hayakawa, S., Puangmanee, S., & Izumori, K. (2006). Chemical properties and antioxidative activity of glycated α-lactalbumin with a rare sugar, D-allose, by Maillard reaction. Food Chemistry, 95(3), 509–517. https://doi.org/10.1016/j.foodchem.2005.01.033

Tavares, T. G., Contreras, M. M., Amorim, M., Martín-Álvarez, P. J., Pintado, M. E., Recio, I., & Malcata, F. X. (2011). Optimisation, by response surface methodology, of degree of hydrolysis and antioxidant and ACE-inhibitory activities of whey protein hydrolysates obtained with cardoon extract. International Dairy Journal, 21(12), 926–933. https://doi.org/10.1016/j.idairyj.2011.05.013

Van der Ven, C., Gruppen, H., de Bont, D. B., & Voragen, A. G. (2002). Optimisation of the angiotensin converting enzyme inhibition by whey protein hydrolysates using response surface methodology. In International Dairy Journal (Vol. 12).

Vaštag, Ž., Popović, L., Popović, S., Krimer, V., & Peričin, D. (2010). Hydrolysis of pumpkin oil cake protein isolate and free radical scavenging activity of hydrolysates: Influence of temperature, enzyme/substrate ratio and time. Food and Bioproducts Processing, 88(2–3), 277–282. https://doi.org/10.1016/j.fbp.2009.12.003

Uluko, H., Zhang, S., Liu, L., Li, H., Cui, W., Xue, H., Zhaoa, L., Sunc, Y., Lua, J., & Lv, J. (2014). Pilot-scale membrane fractionation of ACE inhibitory and antioxidative peptides from ultrasound pretreated milk protein concentrate hydrolysates. Journal of Functional Foods 7 350–361. https://doi.org/10.1016/j.jff.2014.01.025

Zhang, J., Zhang, H., Wang, L., Guo, X., Wang, X., & Yao, H. (2009). Antioxidant activities of the rice endosperm protein hydrolysate: Identification of the active peptide.

European Food Research and Technology, 229(4), 709–719. https://doi.org/10.1007/s00217-009-1103-3

Wickham, H. (2016). ggplot2: Elegant Graphics for Data Analysis. https://ggplot2.tidyverse.org

Wijayanti, H. B., Brodkorb, A., Hogan, S. A., & Murphy, E. G. (2019). Thermal Denaturation, Aggregation, and Methods of Prevention. Whey Proteins, 185–247. doi:10.1016/b978-0-12-812124-5.00006-0

Tables and Figures

Table 1 - Real and coded values of independent variables used in the Box-Behnken Design (BBD).

Experimental variables	Code	Coded level		
		-1	0	1
		Real values		
Temperature (°C)	(A)	20	35	50
Reaction Time (h)	(B)	1	9	17
Enzyme Concentration (%)	(C)	1	3	5

Table 2 – Experimental runs obtained by Box-Behnken design using three independent variables showing observed values of degree of hydrolysis (DH), ABTS+ radical scavenging activity (ABTS) and DPPH radical scavenging activity (DPPH).

Trt	Coded level			Real values			Experimental values					
	A	B	C	T (°C)	Time (h)	Enzyme (%)	Maxipro® PSP			Maxipro® TNP		
							DH (%)	ABTS (%)	DPPH (%)	DH (%)	ABTS (%)	DPPH (%)
1	-1	-1	0	20	1	3	0.33	1.30	3.50	0.52	5.68	5.13
2	1	-1	0	50	1	3	0.48	7.35	7.61	1.04	10.81	9.41
3	-1	1	0	20	17	3	0.47	3.76	5.15	1.55	8.11	8.31
4	1	1	0	50	17	3	1.01	10.29	9.60	3.01	11.14	10.83
5	-1	0	-1	20	9	1	0.48	2.13	3.94	0.80	4.07	5.52
6	1	0	-1	50	9	1	0.67	7.53	8.30	1.49	10.83	10.49
7	-1	0	1	20	9	5	0.51	4.60	5.86	1.53	9.72	9.52
8	1	0	1	50	9	5	0.81	10.51	9.41	5.16	13.17	12.12
9	0	-1	-1	35	1	1	0.41	2.23	1.00	0.61	5.98	8.43
10	0	1	-1	35	17	1	0.48	4.45	3.45	1.15	6.24	4.08
11	0	-1	1	35	1	5	0.37	2.62	7.95	0.70	7.43	8.41
12	0	1	1	35	17	5	1.19	7.46	8.55	2.30	10.61	8.66
13	0	0	0	35	9	3	0.81	5.39	4.27	1.07	7.71	5.97
13	0	0	0	35	9	3	0.64	5.27	4.68	1.08	6.85	4.48
13	0	0	0	35	9	3	0.66	5.72	3.37	0.99	6.36	5.30

Table 3 - ANOVA table showing the terms of each variable and coefficients for predicting the models and goodness of fit statistics.

Enzyme	Responses	Model terms	Coefficient estimate	Standard error	t-value	Pr > |t|	
Maxipro® PSP	DH	Intercept	0.68978	0.04644	14.852	1.5e-06	***
		A	0.14818	0.03418	4.335	0.003416	**
		B	0.19377	0.03418	5.669	0.000759	***
		C	0.10493	0.03418	3.070	0.018069	*
		A^2	-0.06288	0.05016	-1.254	0.250249	
		B^2	-0.06649	0.05016	-1.325	0.226658	
		A×B	0.09628	0.04834	1.992	0.086674	
		B×C	0.18575	0.04834	3.843	0.006353	**
		R^2	*0.9215*				
		R^2 adj	*0.843*				
	ABTS	Intercept	5.4591	0.2491	21.918	1.04e-07	***
		A	2.9849	0.1525	19.571	2.27e-07	***
		B	1.5580	0.1525	10.215	1.86e-05	***
		C	1.1080	0.1525	7.265	0.000168	***
		A^2	1.1086	0.2245	4.938	0.001679	**
		B^2	-0.8935	0.2245	-3.980	0.005324	**
		C^2	-0.3754	0.2245	-1.672	0.138387	
		B×C	0.6539	0.2157	3.032	0.019072	*
		R^2	*0.9884*				
		R^2 adj	*0.9768*				
	DPPH	Intercept	4.3252	0.5706	7.579	3.4e-05	***
		A	2.0579	0.4200	4.900	0.000848	***
		B	0.8368	0.4200	1.992	0.077503	
		C	1.8834	0.4200	4.485	0.001522	**
		A^2	1.9756	0.6164	3.205	0.010741	*
		C^2	0.7435	0.6164	1.206	0.258473	
		R^2	*0.8683*				
		R^2 adj	*0.7951*				
Maxipro® TNP	DH	Intercept	0.8727	0.2856	3.056	0.01569	*
		A	0.7870	0.2102	3.744	0.00567	**
		B	0.6417	0.2102	3.053	0.01575	*
		C	0.7034	0.2102	3.347	0.01013	*
		A^2	0.7891	0.3085	2.558	0.03375	*
		C^2	0.4500	0.3085	1.459	0.18271	
		A×C	0.7357	0.2973	2.475	0.03841	*
		R^2	*0.8593*				
		R^2 adj	*0.7537*				
	ABTS	Intercept	7.0009	0.2849	24.575	2.99e-07	***
		A	2.2976	0.2097	10.958	3.43e-05	***
		B	0.7737	0.2097	3.690	0.010209	*
		C	1.7271	0.2097	8.237	0.000173	***
		A^2	1.9195	0.3077	6.238	0.000786	***
		C^2	0.5455	0.3077	1.773	0.126653	
		A×B	-0.5253	0.2965	-1.772	0.126854	
		A×C	-0.8274	0.2965	-2.790	0.031557	*
		B×C	0.7319	0.2965	2.468	0.048576	*
		R^2	*0.9774*				
		R^2 adj	*0.9472*				
	DPPH	Intercept	5.60569	0.63985	8.761	2.26e-05	***
		A	1.79567	0.47092	3.813	0.00514	**
		B	0.06391	0.47092	0.136	0.89540	
		C	1.27339	0.47092	2.704	0.02690	*
		A^2	2.54956	0.69112	3.689	0.00614	**
		C^2	1.52345	0.69112	2.204	0.05860	
		B×C	1.15307	0.66598	1.731	0.12162	
		R^2	*0.8408*				
		R^2 adj	*0.7215*				

Significance codes: '***' 0.001 '**' 0.01 '*' 0.05

Table 4 - Second-order polynomial equations.

Response parameters			Second-order polynomial model equation
Maxipro ® PSP	DH	Coded	0.6898 + 0.1482 A + 0.1938 B + 0.1049 C – 0.0629 A^2 – 0.0665 B^2 + 0.0963 AB + 0.1857 BC
		Uncoded	0.1083 + 0.0222 A – 0.0200 B – 0.0520 C – 0.0003 A^2 - 0.0010 B^2 + 0.0008 AB + 0.0116 BC
	ABTS	Coded	5.4591 + 2.9849 A + 1.5580 B + 1.1080 C + 1.1086 A^2 – 0.8935 B^2 – 0.3754 C^2 + 0.6539 BC
		Uncoded	0.2428 – 0.1459 A + 0.3234 B + 0.7493 C + 0.0049 A^2 – 0.0140 B^2 – 0.0938 C^2 + 0.0409 BC
	DPPH	Coded	4.3252 + 2.0579 A + 0.8368 B + 1.8834 C + 1.9756 A^2 + 0.7435 C^2
		Uncoded	8.1858 – 0.4774 A + 0.1046 B – 0.1735 C + 0.0088 A^2 + 0.1859 C^2
Maxipro ® TNP	DH	Coded	0.8727 + 0.7870 A + 0.6417 B + 0.7034 C + 0.7891 A^2 + 0.4500 C^2 + 0.7357 AC
		Uncoded	5.1431 – 0.2666 A + 0.0802 B – 1.1817 C + 0.0035 A^2 + 0.1125 C^2 + 0.0245 AC
	ABTS	Coded	7.0009 + 2.2976 A + 0.7737 B + 1.7271 C + 1.9195 A^2 + 0.5455 C^2 – 0.5253 AB – 0.8274 AC + 0.7319 BC
		Uncoded	6.8167 – 0.3218 A + 0.1127 B + 0.5990 C + 0.0085 A^2 + 0.1364 C^2 – 0.0044 AB – 0.0276 AC + 0.0457 BC
	DPPH	Coded	5.6057 + 1.7957 A + 0.0639 B + 1.2734 C + 2.5496 A^2 + 1.5234 C^2 + 1.1530 BC
		Uncoded	18.6883 – 0.6735 A – 0.2082 B – 2.2971 C + 0.0113 A^2 + 0.3809 C^2 + 0.0721 BC

Where A and A^2 are the linear and square for temperature, B and B^2 are the linear and square term for reaction time, C and C^2 are the linear and square terms for enzyme concentration, AB is the interaction term for temperature and reaction time, BC is the interaction term for reaction time and enzyme concentration and AC is the interaction term for temperature and enzyme concentration.

Table 5 - Optimization of the response parameters.

Response parameters		Coded			Uncoded			Response (%)
		Temp (°C)	Time (h)	Enzyme (%)	Temp (°C)	Time (h)	Enzyme (%)	
Maxipro® PSP	DH	0.5489	0.9385	0.9044	43.2338	16.5081	4.8087	1.1776
	ABTS	1.0000	0.6622	0.7493	50.0000	14.2976	4.4987	11.1365
	DPPH	1.0000	0.2440	0.9698	50.0000	10.9521	4.9395	11.0885
Maxipro® TNP	DH	1.0000	0.2680	0.9634	50.0000	11.1444	4.9268	4.4249
	ABTS	1.0000	0.3884	0.9215	50.0000	12.1073	4.8430	12.8687
	DPPH	1.0000	0.2529	0.9675	50.0000	11.0230	4.9350	12.9072

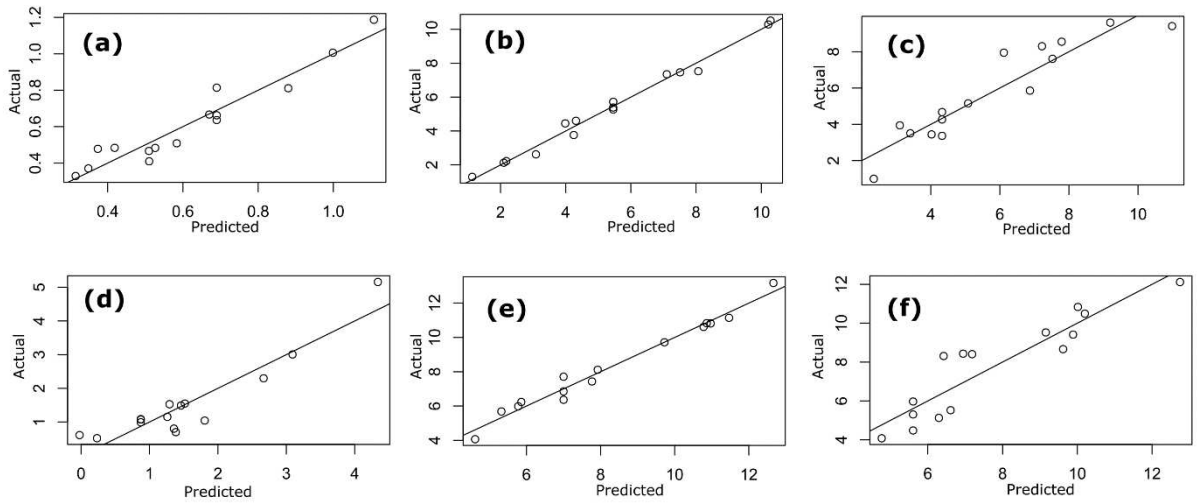

Figure 1 - The plot of predicted versus actual values when using enzyme Maxipro® PSP (a, b, c) and using Maxipro® TNP (d, e, f) for (a, d) degree of hydrolysis (DH) response, (b, e) antioxidant activity (ABTS methodology) response, (c, f) antioxidant activity (DPPH methodology) response.

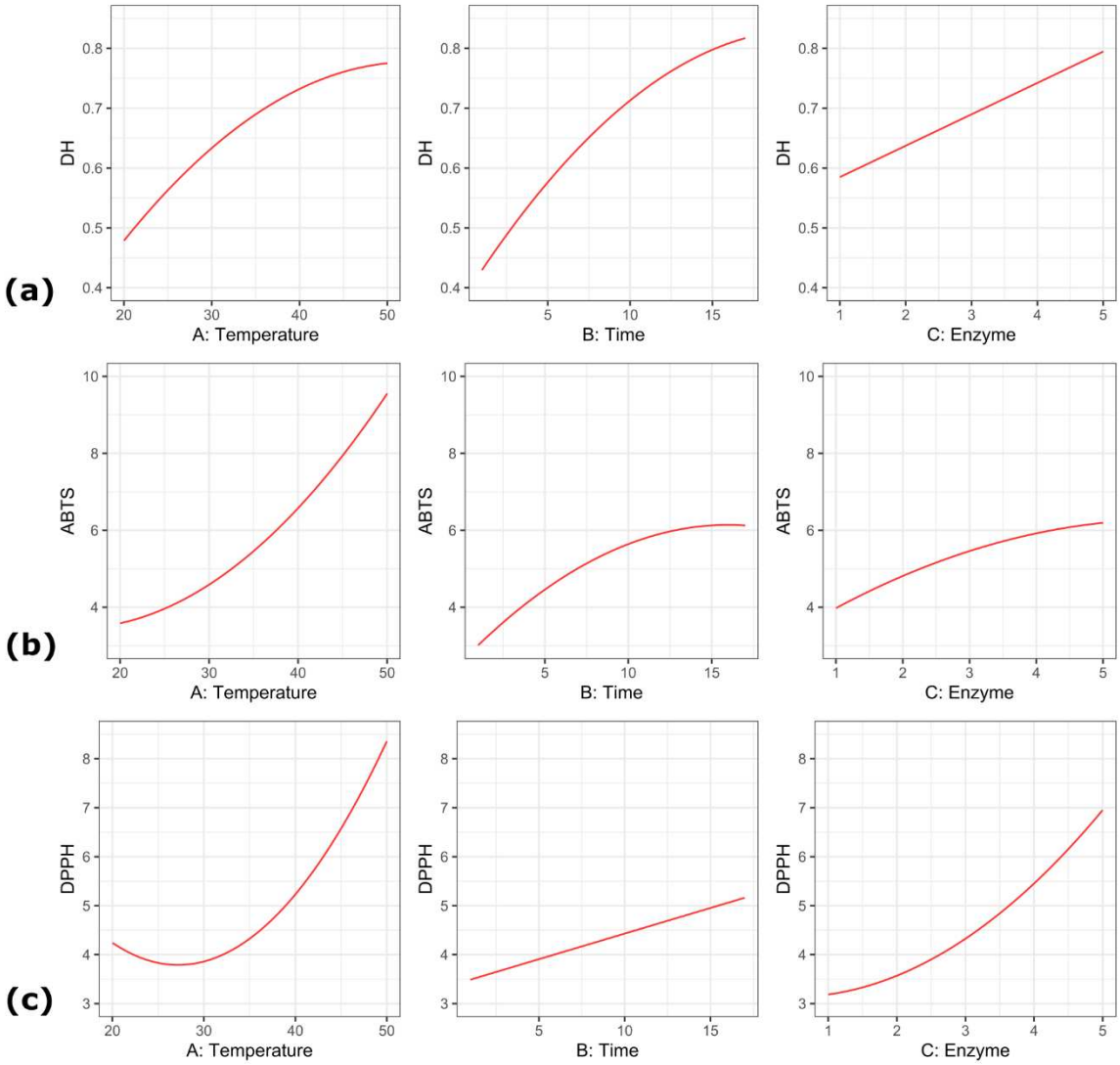

Figure 2 - Individual effects plot. Maxipro® PSP. (a) Degree of hydrolysis (DH) response. (b) Antioxidant activity (ABTS methodology) response. (c) Antioxidant activity (DPPH methodology) response.

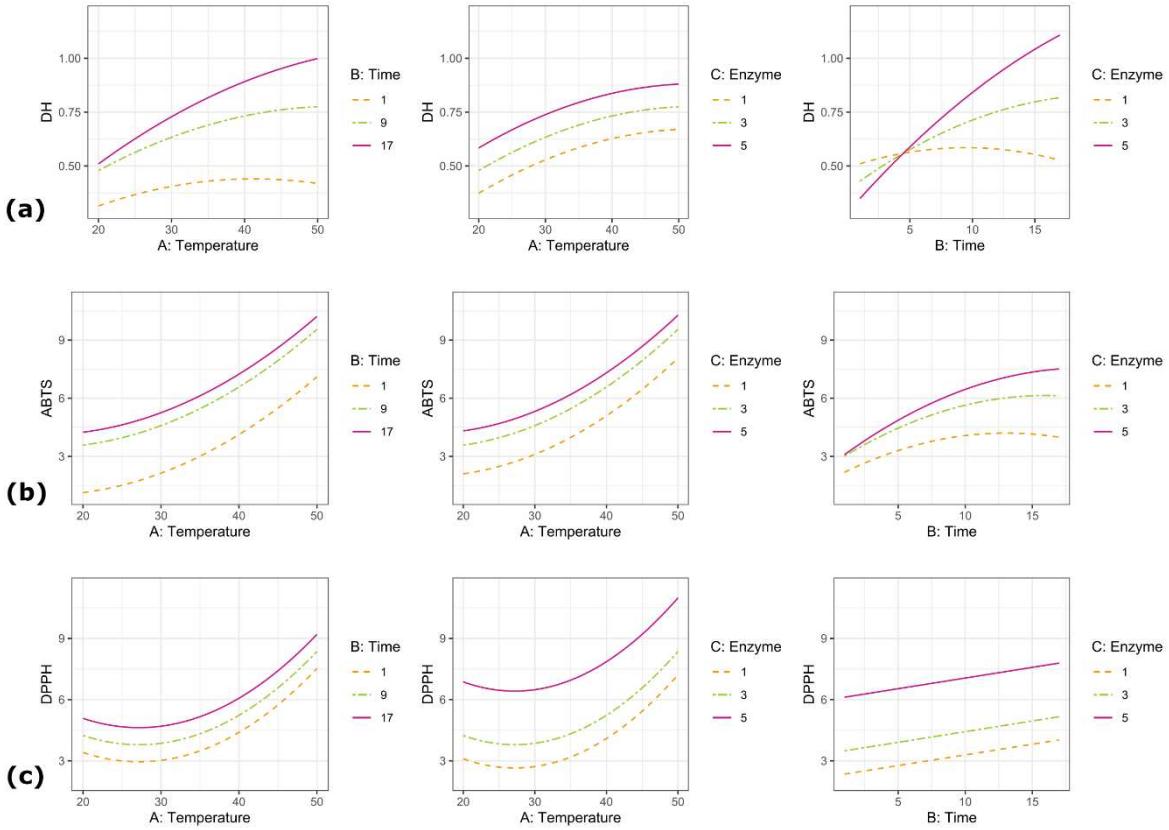

Figure 3 - Interaction plots. Maxipro® PSP. (a) Degree of hydrolysis (DH) response. (b) Antioxidant activity (ABTS methodology) response. (c) Antioxidant activity (DPPH methodology) response.

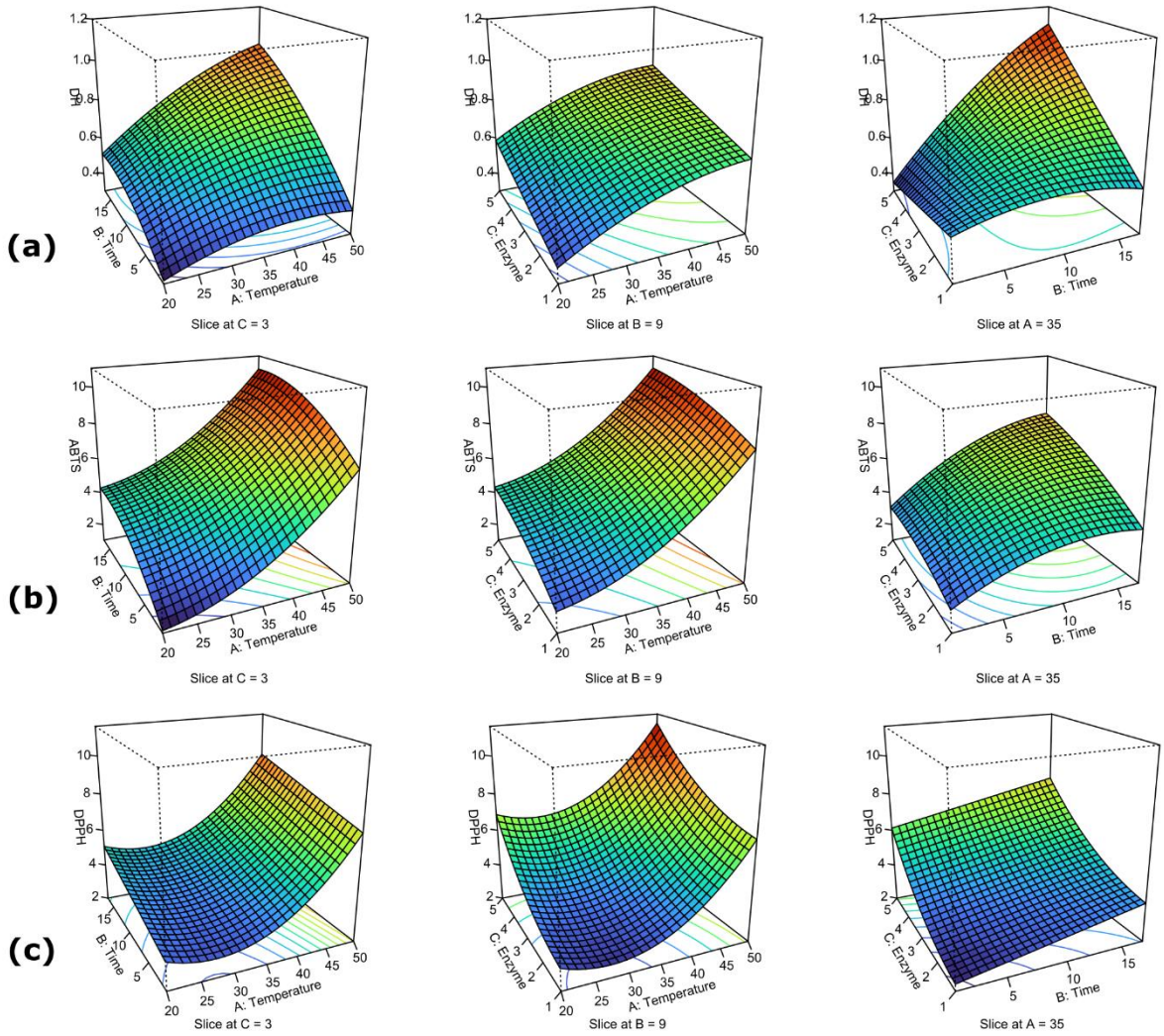

Figure 4 - Response surface plots (3D). Maxipro® PSP. (a) Degree of hydrolysis (DH) response. (b) Antioxidant activity (ABTS methodology) response. (c) Antioxidant activity (DPPH methodology) response.

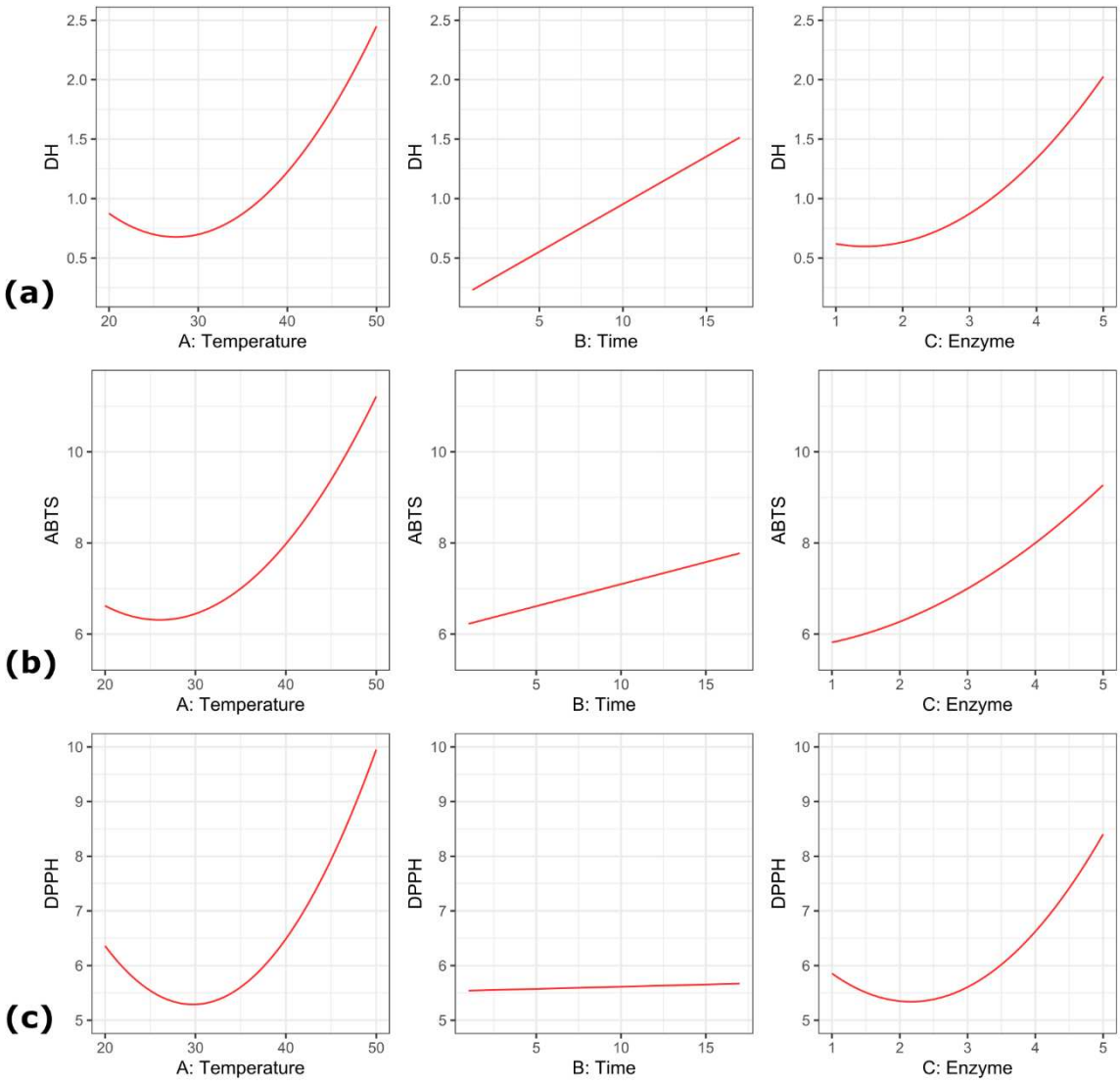

Figure 5 - Individual effects plot. Maxipro® TNP. (a) Degree of hydrolysis (DH) response. (b) Antioxidant activity (ABTS methodology) response. (c) Antioxidant activity (DPPH methodology) response.

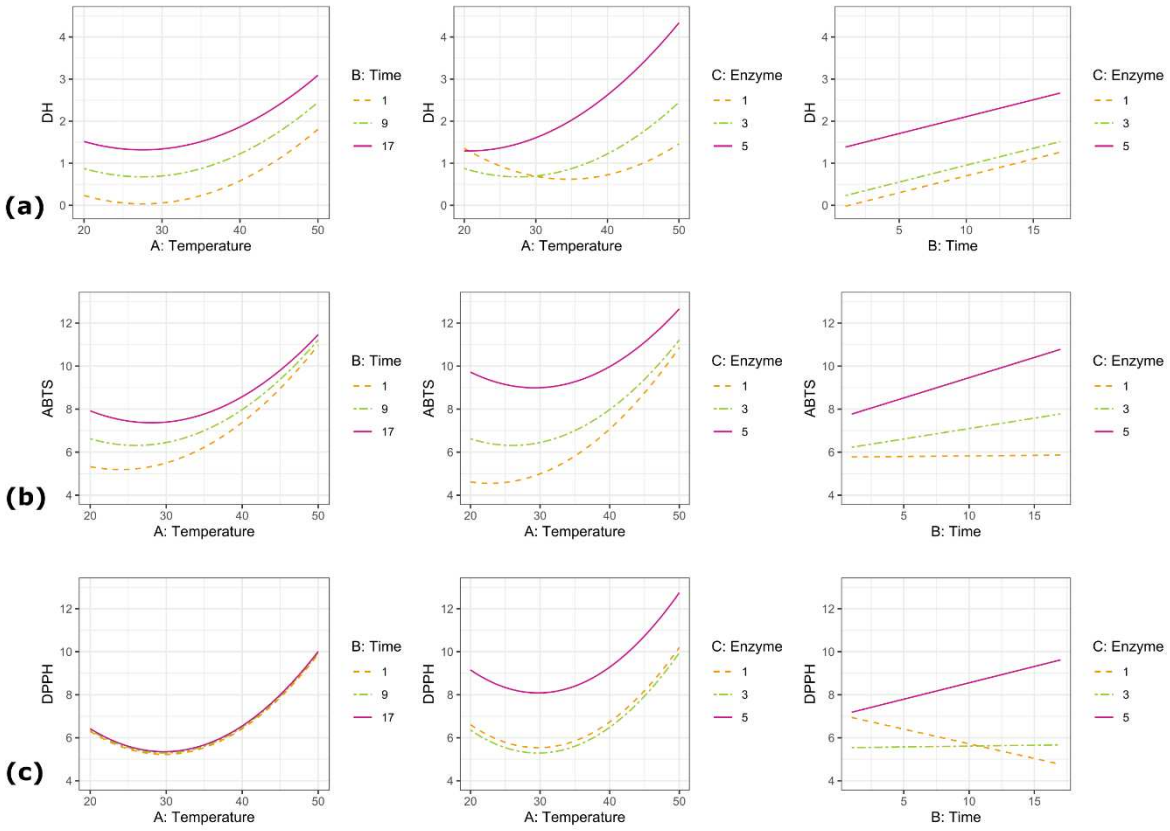

Figure 6 - Interaction plots. Maxipro® TNP. (a) Degree of hydrolysis (DH) response. (b) Antioxidant activity (ABTS methodology) response. (c) Antioxidant activity (DPPH methodology) response.

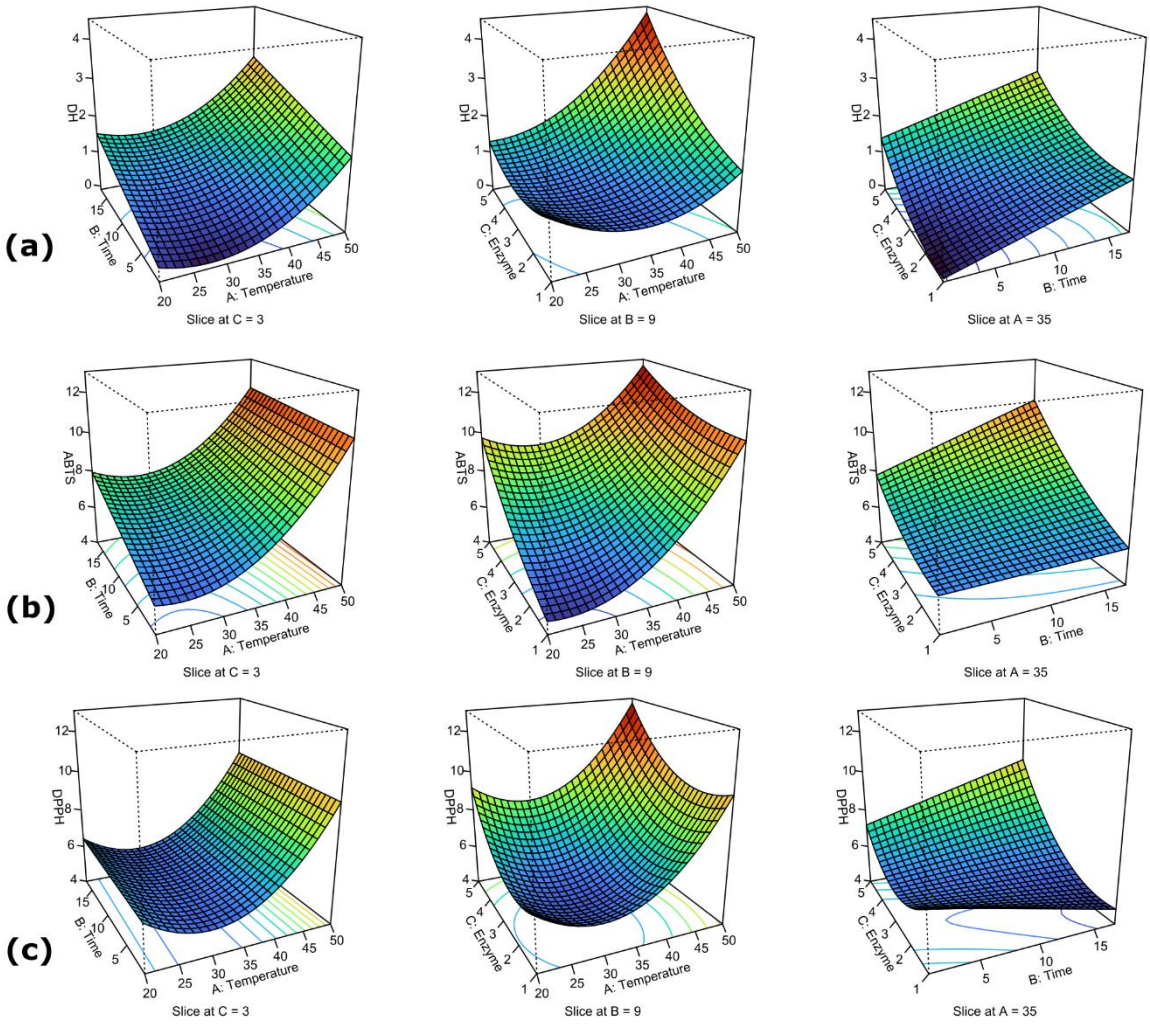

Figure 7 - Maxipro® TNP. (a) Degree of hydrolysis (DH) response. (b) Antioxidant activity (ABTS methodology) response. (c) Antioxidant activity (DPPH methodology) response.

4. General conclusions and perspectives

Peptidases presented consolidated applications in dairy technology, currently used to improve bio and techno-functional features of milk proteins and in the development of dairy products. As discussed in Chapter 1, the growing application of peptidases is dependent on diverse points that comprise the selection of the most suitable enzyme, taking into count its specificity, the protein source used, and the hydrolyzed products aimed. For future peptidases applications and improved performances that are used today, it is important to include the research of new enzymes, as well as prior hydrolysis processes to facilitate peptidases action, and study the obtention of hydrolysates with high-added value, including bioactive peptides. Moreover, hydrolysis conditions need to be properly determined to prevent the disapproving sensory effects, such as bitter-flavored, and also to obtained the aimed hydrolysates.

In Chapter 2 optimization by RSM showed as an effective tool to set the best hydrolysis parameters (temperature, time, and enzyme concentration) aimed at the release of whey protein hydrolysates with antioxidant activity. These hydrolysates are presented as potential antioxidant ingredients; however, studies are needed to ensure this bioactivity. In this way, a proposal for further studies is to characterize the profile of those whey protein hydrolysate sequences responsible for antioxidant activity, and also to check this bioactivity in vivo.