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Review

Plant proteases and their application in dairy systems

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ABSTRACT

Enzymatic hydrolysis of proteins is considered a feasible approach to obtain more functional and nutritional products. Plant proteases (either purified or as crude extracts) have been used in dairy systems with growing interest. Specific plant proteases such as actinidin, bromelain, ficin and papain have been isolated and extensively characterised. Their application on dairy proteins can provide benefits by providing a product that is less allergenic or with improved techno-functionality. Also, benefits can include hydrolysates with reduced bitterness and obtaining of bioactive peptides with enhanced nutritional and physiological properties. This review describes the use of plant proteases in hydrolysis, application of specific proteases in dairy applications.

Contents

1. Introduction

Milk proteins present a diverse group of proteins, composed of the caseins and whey proteins. In addition to being nutritionally very valuable, a major role of caseins in food applications is often as a structure builder. Caseins are a versatile group of proteins consisting of 4 types named α_{s1}-, α_{s2}-, β- and κ-casein (CN) (Huppertz, 2013). On the other hand, whey proteins are considered valuable due to an abundance of branched chain amino acids (BCAA), which play a crucial role for e.g., muscle physiology (Sah, McAinch, & Vasiljevic, 2016). Whey proteins in commercially available formats, such as in the form of whey protein concentrates or isolates, are usually derived from co-products in the production of majority of cheeses and caseinates. Whey proteins are a very diverse group of proteins, including α-lactalbumin (α-LA), β-lactoglobulin (β-LG), bovine serum albumin (BSA), lactoferrin, lactoperoxidase, and various immunoglobulins (Dupont, Croguennec, Brodkorb, & Kouaouci, 2013). In addition, whey protein derived peptides possess various physiologically important properties impacting the immune, cardiovascular, digestive and nervous systems (Ghosh, Prasad, & Saha, 2017). However, their application in food systems

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can be hindered with several important issues such as cow milk protein allergies (CMPA) and poor stability during processing as most prevalent (Host & Halken, 2014). Therefore, efficient utilization of milk proteins in food systems may depend on tailoring their structural characteristics.

Protein modifications can be achieved by various chemical and biochemical methods, such as use of acids or alkali or by microbial or enzymatic hydrolysis (Ovissipour et al., 2013). In the food industry, the former techniques have disadvantages. Acid treatment may not be preferred as it converts Gln to Glu and Asn to Asp, partially destroys Ser and Thr, and also causes oxidation of Met and Cys, whereas treatment with alkali can cause racemization of amino acids. Therefore, hydrolysates of milk proteins are usually obtained through enzymatic hydrolysis as this provides the advantages of fast reaction rates, mild processing conditions and high specificity compared to the chemical methods (Noman et al., 2018). Because of the relatively low value of (some) food ingredients, processing enzymes must be cheap relative to total costs. Furthermore, enzymes may be highly substrate or site specific, so that a number of enzymes may be needed to achieve required modification(s) (Krem, Rose, & Di Cera, 2000).

A wide range of proteases (EC 3.4) exists in nature, with different functions and specificities (Krem et al., 2000). Protease specificity is governed by the way the protease interacts with the substrate to perform its action; this is the core of protease applications and thus can reflect on the properties of the final product (Krem et al., 2000). According to the Enzyme Commission (EC), proteases are classified into group 3 (hydrolases) and subgroup 4 (hydrolysis of peptide bonds); however, they can also be classified according to the origin (animal, plant or microbial), catalytic action (endo or exopeptidase), molecular size, active site, charge and substrate specificity (Sumantha, Larroche, & Pandey, 2006). Enzymes from plant, animal, and microbial origin, such as papain, bromelain, ficin, actinidin, alcalase, pepsin, trypsin, chymotrypsin, are among commercially available proteases that have been used to produce milk protein hydrolysates (Rawlings, Barrett, Woessner, & Salvesen, 2012).

Plant proteases are fast emerging and feasible approach from the industrial point of view due to their easy accessibility; they are more economical than proteases from other sources, usually high proteolytic activity and unique characteristics in terms of their stability at high temperatures which allows for a better controlled process without destroying any essential amino acids (Gurumallesh, Alagu, Ramakrishnan, & Muthusamy, 2019). Protease specificity is governed by the way it interacts with the substrate to perform its action, which is the core of protease applications and thus can be invariably reflected on the properties of the final product (Krem et al., 2000). Understanding how proteases perform their functions and under which conditions is important in the search for appropriate and new enzymes and is the aim of this review.

2. Plant proteases and their characteristics

Initially plant proteases were mainly used in the form of plant tissues and crude, usually aqueous, extracts while nowadays, due to advancements in extraction, purification and characterisation techniques, they are also used in a pure form (Tavano, Berenguer-Murcia, Secundo, & Fernandez-Lafuente, 2018). Plant-based proteases are now widely produced, either extracted directly from their natural source or produced through cultures (Table 1), such as papain (EC 3.4.22.2), bromelain (EC 3.4.22.32–33), ficin (EC 3.4.22.3) and actinidin (EC 3.4.22.14) (Table 2) (Rawlings et al., 2012).

Plant-derived cysteine proteases are divided into five clans, CA, CD, CE, CF and CO, with majority of proteases belonging to clan CA (Papain family) (Feijoo-Siota & Villa, 2011). Proteases from the CA clan must have a targeting sequence, to direct them to a specific cellular compartment, and the cleavage of a protein precursor, to activate the enzyme, at the N-terminus of the enzyme. An extensive homology has been found in the amino acid sequence, substrate specificity and tertiary structure of all members of CA clan, the C1 family (Baker, Boland, Calder, & Hardman, 1980; Carne & Moore, 1978). Their structure consists of a β-barrel like and a α-helix disunited by a grove, consisting of the active site with Cys_{25} and His_{159} residues on each side of the groove (Fig. 1) and are present in all enzymes of the family. Asp_{175}, which orients the His_{159} ring, and Glu_{19}, which leads to Cys_{25}, are two additional residues that are also crucial for catalysis of CA family (Carne & Moore, 1978; Feijoo-Siota & Villa, 2011).

Actinidin is a cysteine protease consisting of 220 amino acids and has a molecular weight of 23.5 kDa. Actinidin is extracted from kiwifruit and is active in the pH range of 4–10 and the temperature range 15–60 °C (Baker et al., 1980; Zhu, Kaur, & Boland, 2018). The actinidin amino acid sequence contains a total of seven Cys residues, with one is located inside the active site and the other six are involved in the formation of three disulphide bridges. The polypeptide chain of actinidin is folded into α-helices and twisted β-sheets, where the α-helix domain consists of residues from f(19–115) and f(214–218) and the β-sheets contain residues f(1–18) and f(116–213). This type of folding arrangement leads to the protein cleavage occurring in between both domains. The amino end of one domain is linked with carboxylic group of another domain, which leads to formation of a belt-like structure and hence exhibit actinidin stability. Cys_{25} and His_{162} are two residues present at the active site behind the cleft in the middle of the domains of actinidin (Grozdanović, Gavrović-Jankulović, & Drakulić, 2013; Rawlings et al., 2012). An active site of actinidin consists of seven subsites (S1, S2, S3, S4, S1′, S2′ and S3′) that bind with an amino and carboxylic end of the side chain of an amino acid of the substrate (P1, P2, P3, P4, P1′, P2′ and P3′) (Baker et al., 1980; Boland & Singh, 2013). The interaction of subsite S2 towards P2 of the substrate provides major contribution towards actinidin specificity. S2 subsite of actinidin mainly consists of side chains of Tyr_{67}, Ile_{70}, Thr_{69}, Ser_{205}, Met_{211}, Val_{133} and Val_{157}. In actinidin, Met_{211} is present at the lower part of binding pocket of the S2 subsite, but its side chain changes position during creation of an actinidin substrate complex, which completely allows sidechains of Phe residue to approach S2 subsite (Baker et al., 1980; Boland & Singh, 2013; Rawlings et al., 2012). Actinidin mostly cleaves amino acids present on hydrophobic sites of the P2 residue, such as Leu, Val or Phe (Boland & Singh, 2013).

Papain contains 212 amino acids and has a molecular weight of 23.4 kDa. It has three disulphide bridges and one free sulfhydryl group. Papain cleaves the peptides containing amino acids Ala, Ile, Trp, Phe, Val, Leu and Tyr (Lorenzo et al., 2018). Similar to actinidin, papain exhibits a preference for an amino acid with a large hydrophobic side chain at the P2 position; however, unlike actinidin, papain does not accept Val at the P1′ position (Lorenzo et al., 2018).

Bromelain resembles papain and actinidin in terms of substrate specificity and it mainly cleaves after Lys, Ala, Tyr and Gly (Rawlings et al., 2012). Its optimum pH is 6.0–8.5 and its optimum temperature is 50–60 °C. Bromelain is obtained from the stem and fruit of the pineapple. Fruit bromelain exhibits broader specificity and higher proteolytic activity as compared to stem bromelain (Polaina & MacCabe, 2007).

Similarly, ficin (EC 3.4.22.3) exhibits optimum pH range of 5.0–8.0 and temperature is 45–55 °C (Polaina & MacCabe, 2007). For ficin, only N-terminus (His and Cys) sequencing has been studied so far and Cys showed homology to that of papain sequencing. Furthermore, a study conducted by Devaraj, Gowda, and Prakash (2008) revealed enzymatic specificity of ficin towards hydrolysing peptide bonds C-terminal to Glu, Leu and Phe at the P_1 position.

Table 1
Examples of plant proteases commercially produced by in vitro techniques.

Species	Protease	Type of culture	Reference
Ananas comosus	Bromelain	Micropropagation, callus	Fernandez and Pomilio (2003)
Actinidia deliciosa	Actinidin	Micropropagation	Nadarajan et al. (2023); Prado, Herrera, Vázquez, Romo, and González (2005); Wu (2017)
Ficus carica	Ficin	Micropropagation, cell suspension, callus	Dini et al. (2021); Gupta, Jain, Joseph, and Devi (2020); Kim and Li-Chan (2006); Pasqual and Ferreira (2007);
Taxus canadensis	Peptidase extract	Micropropagation	Gupta et al. (2020)
Hypericum perforatum	Peptidase extract	Micropropagation	Gupta et al. (2020)
Cynara cardunculus	Cardosin	Cell suspension, callus	Anandan, Sudhakar, Balasubramanian, and Gutiérrez-Mora (2012); Elateeq, Sun, Nxumalo, and Gabr (2020); Folgado, Pires, Figueiredo, Pimentel, and Abranches (2020)
Silybum marianum	Silymarin	Cell suspension, callus	Anandan et al. (2012); Cimino, Cavalli, Spina, Natalucci, and Priolo (2006); Elateeq et al. (2020); Folgado et al. (2020)
Carica papaya	Papain	Micropropagation, callus	Gupta et al. (2020); Panjaitan, Aziz, Rashid, and Saleh (2007)
Coleus forskohlii	Forskolin	Micropropagation	Gupta et al. (2020)

Table 2
Main plant derived endopeptidases (proteinases) used in dairy systems.

Proteinase type	Proteinase name	References
Cysteine	Papain-like	Gavira, Gonzalez-Ramirez, Oliver-Salvador, Soriano-Garcia, and Garcia-Ruiz (2007); Torres et al. (2010)
	Papain	Abe, Wu, Kim, Fujii, and Abe (2015); Fernández-Lucas, Castañeda, and Hormigo (2017); Kaur et al. (2023b); Mahajan and Chaudhari (2014).
	Bromelain	Arshad et al. (2014); Kaur et al. (2023b)
	Ficin	Morellon-Sterling, El-Siar, Tavano, Berenguer-Murcia, and Fernández-Lafuente (2020)
	Actinidin	Grozdanovic, Burazer, and Gavrovic-Jankulovic (2013); Kaur et al. (2021); Kaur et al. (2023b); Zhang, Sun, Liu, Li, and Jiang (2017)
Serine	Dubiumin	Ahmed, Morishima, Babiker, and Mori (2009)
	Subtilisins	Asif-Ullah, Kim, and Yu (2006); Laplaze et al. (2000); Uchikoba et al. (2001)
	Latex glycoprotein (LGP)	Rajesh et al. (2006)
	Religiosin	Kumari, Sharma, and Jagannadham (2010)
	Milin	Yadav, Pande, and Jagannadham (2006)
	Neriifolin	Yadav, Patel, and Jagannadham (2012)
Aspartic	Asteraceae	Raposo and Domingos (2008)
	Cyprosins and cardosins	Liburdi, Spinelli, Benucci, Lombardelli, and Esti (2018); Mazorra-Manzano et al. (2013)
	Onopordosin	Brutti, Pardo, Caffini, and Natalucci (2012)
	Arctiumisin	Cimino, Colombo, Liggieri, Bruno, and Vairo-Cavalli (2015)
	Purified extract from *Centaurea calcitrapa*	Raposo and Domingos (2008)
	Protein extract from *Ficus racemosa latex*	Devaraj et al. (2008)
	Purified extract *Withania coagulans*	Salehi, Aghamaali, Sajedi, Asghari, and Jorjani (2017)
	Purified extract *Foeniculum vulgare*	Bey, Debbebi, Abidi, Marzouki, and Salah (2018)

3. Improving properties of milk protein hydrolysates

Activity of any enzyme is influenced by several important factors including substrate concentration, pH, ionic strength, nature of ionic environment and temperature (Kaur, Huppertz, & Vasiljevic, 2021; Palmer, 2001). Furthermore, kinetic characterisation of enzymes with specific substrate is also a crucial step for best estimation of selection of that enzyme from industrial production point of view (Kaur, Vasiljevic, & Huppertz, 2023b). So, optimization of processing conditions with correct choice of enzyme can lead to achievement of beneficial properties by exerting changes in peptide/amino acid conformations (Tavano et al., 2018).

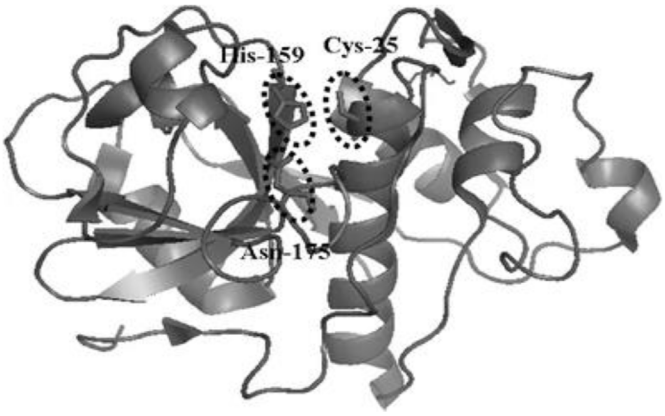

Fig. 1. Three-dimensional model of cysteine protease (papain), PDB code: 1PPN (adapted from Pickersgill, Harris, & Garman, 1992).

3.1. Alteration in allergenicity

Despite of their versatility, cows' milk proteins are considered among the so-called "Big-8" food allergens (Bogahawaththa, Chandrapala, & Vasiljevic, 2017). Cows' milk protein allergy (CMPA) is the most prevalent food allergy among infants. Cows' milk contains approximately 35 allergen proteins, with many present in trace amounts. Some of the major milk proteins epitopes regions and their amino acid sequences can be found in Table 3. One of the approaches in the management of CMPA is based on avoidance of milk proteins in the diet, but this may have substantial consequences on a person's development due to lack of appropriate intake of essential amino acids. However, the allergic properties of many proteins can also be reduced by enzymatic hydrolysis. Hydrolysis by plant proteases assists in minimizing protein allergenicity by converting proteins to peptides and free amino acids, as a result of which reactive epitopes may no longer be recognized by

Table 3
Some of the major milk proteins epitopes regions along with their amino acid sequences.

Protein fraction	Epitopes	Specific amino acid sequence	References
αs_1-CN	f(21–35)	Leu-Arg-Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys-Glu	Cong, Yi, Qing, and Li (2013)
	f(56–70)	Asp-Ile-Lys-Gln-Met-Glu-Ala-Glu-Ser-Ile-Ser-Ser-Ser-Glu-Glu	
	f(161–175)	Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp	
β-CN	f(1–14)	Arg-Glu-Leu-Glu-Glu-Leu-Asn-Val-Pro-Gly-Glu-Ile-Val-Glu	Chatchatee et al. (2001)
	f(23–36)	Ile-Thr-Arg-Ile-Asn-Lys-Lys-Ile-Glu-Lys-Phe-Gln	
	f(55–69)	Thr-Gln-Ser-Leu-Val-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn	
	f(81–94)	Gln-Thr-Pro-Val-Val-Val-Pro-Pro-Phe-Leu-Gln-Pro-Glu-Val	
	f(107–122)	Lys-Glu-Met-Pro-Phe-Pro-Lys-Tyr-Pro-Val-Glu-Pro-Phe-Thr	
	f(135–144)	Leu-Pro-Leu-Pro-Leu-Leu-Gln-Ser-Trp-Met	
	f(149–162)	Gln-Pro-Leu-Pro-Pro-Thr-Val-Met-Phe-Pro-Pro-Gln	
	f(170–182)	Lys-Val-Leu-Pro-Val-Pro-Gln-Lys-Ala-Val-Pro-Tyr-Pro-Gln	
	f(183–208)	Arg-Asp-Met-Pro-Ile-Gln-Ala-Phe-Leu-Leu-Tyr-Gln-Glu-Pro-Val-Leu-Gly-Pro-Val-Arg-Gly-Pro-Phe-Pro-Ile-Ile	
κ-CN	f(15–24)	Glu-Arg-Phe-Phe-Ser-Asp-Lys-Ile-Ala-Lys	Chatchatee et al. (2001)
	f(38–47)	Ser-Tyr-Gly-Leu-Asn-Tyr-Tyr-Gln-Gln-Lys	
	f(55–81)	Phe-Leu-Pro-Tyr-Pro-Tyr-Tyr-Ala-Lys-Pro-Ala-Ala-Val-Arg-Ser-Pro-Ala-Gln-Ile-Leu-Gln-Trp-Gln-Val	
	f(105–117)	Phe-Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys	
β-LG	f(41–60)	Val-Tyr-Val-Glu-Glu-Leu-Lys-Pro-Thr-Pro-Glu-Gly-Asp-Leu-Glu-Ile-Leu-Leu-Gln-Lys	Bogahawaththa et al. (2017); Fox (2003)
	f(102–124)	Tyr-Leu-Leu-Phe-Cys(forms disulphide bridge with Cys_{119})-Met-Glu-Asn-Ser-Ala-Glu-Pro-Glu-Gln-Ser-Leu-Ala-Cys (forms disulphide bridge with Cys_{106})-Gln-Cys (a free thiol group)-Leu-Val-Arg (very stabilised sequence)	
	f(149–162)	Leu-Ser-Phe-Asn-Pro-Thr-Gln-Leu-Glu-Glu-Gln-Cys (forms disulphide bridge with Cys_{66})-His-Ile (makes flexible turns at the hydrophobic carboxyl terminus)	
α-LA	f(7–18)	Glu-Val-Phe-Arg-Glu-Leu-Lys-Asp-Leu-Lys-Gly-Tyr	Järvinen, Chatchatee, Bardina, Beyer, and Sampson (2001)
	f(53–62)	Phe-Gln-Ile-Asn-Asn-Lys-Ile-Met-Cys-Lys	
	f(89–108)	Ile-Met-Cys-Val-Lys-Lys-Ile-Leu-Asp-Lys-Val-Gly-Ile-Asn-Tyr-Trp-Leu-Ala-His-Lys	

antibodies that would initiate allergenic reaction (Noman et al., 2018).

Cysteine plant proteases have shown higher effectiveness with dairy proteins compared to proteases from other sources. For example, a study by Izquierdo, Peñas, Baeza, and Gomez (2008) on enzymatic hydrolysis of WPC under microwave irradiation by using pronase, chymotrypsin, corolase, alcalase, neutrase or papain showed that treatment with papain resulted in the largest immunoreactivity reduction after alcalase, whereas treatment with pronase was slightly lower than papain; hydrolysis with chymotrypsin, corolase and neutrase resulted in negligible immunoreactivity reduction. Also, papain (Liang et al., 2020), actinidin (Kaur, Huppertz, & Vasiljevic, 2022), ficin (Aider, 2021) and bromelain (Hasegawa et al., 2017) have been used on different milk proteins aimed to reduce antigenicity of milk proteins, and significant reductions were observed.

Infant formulas containing protein hydrolysates differ due to protein source, the degree of hydrolysis and the profiles of released peptides, all of which are enzyme dependant (type) and other pre- and post-processing methods (Exl & Fritsche, 2001). Table 4 shows selected milk protein hydrolysates obtained by treatment with plant proteases, resulting in significant reduction in antigenicity and allergenicity. For example, IgE immunoreactivity of WPI was reduced by 47% after treatment with papain when hydrolysis performed at optimum conditions for 5 h at enzyme to substrate ratio of 1–100 (Zadeh, 2017). Furthermore, in a study of actinidin hydrolysis with milk protein concentrate (MPC) and whey protein isolate (WPI), significant reductions in the antigenicity of β-LG (43%) and αs_1-CN (48%) for MPC and β-LG (54%) for WPI at 60 °C occurred (Kaur et al., 2022). Hydrolysis at 10 °C also resulted in reduction in antigenicity of β-LG (39%) and αs_1-CN (42%) for MPC and β-LG (14%) for WPI (Kaur et al., 2022). Izquierdo et al. (2008) also reported a significant decrease in immunoreactivity in WPC hydrolysates obtained by papain treatment, whereas Villas-Boas, Benedé, de Lima Zollner, Netto, and Molina (2015) showed β-LG hydrolysates obtained by bromelain resulted in reduction of the number of epitopes and the IgE-binding capacity of native β-LG. A study conducted by Liang et al. (2020) indicated that cow milk treated with papain showed a %DH of only 4.5. However, this DH still showed significant reduction in IgG reactivity (75% reduction). Also, an animal study showed that the cow's milk proteins hydrolysed for 24 h by *Carica papaya* exhibited no immune reactions in mice allergic to cow's milk (Oliveira et al., 2019).

Combinations of enzymes can also be used to achieve extensive hydrolysis. For instance, a significant reduction of antigenicity was observed after two step hydrolysis of WPC with alcalase and papain, but immunoreactive epitopes still remained present (Wróblewska & Troszyńska, 2005). In another study, peptides of freeze-dried demineralized cheese whey with 40% reduced antigenicity were obtained by hydrolysis with combinations of papain and trypsin (Shin et al., 2007).

While numerous studies have been conducted, the research is still ongoing to obtain allergen-free milk proteins hydrolysates (Freidl et al., 2022; Fritsché, 1998; Pecquet, Bovetto, Maynard, & Fritsché, 2000). Thus, further studies are needed with a multitude of objectives including enzyme selection and establishment of processing conditions that would result in hypoallergenic hydrolysates.

3.2. Changes in techno-functional properties

Limitations with functional properties of some dairy proteins can limit their use in some applications. For example, high viscosity or poor solubility of MPC/MPI at room temperature and neutral pH can limit utilisation in high energy drinks (Havea, 2006; Singh, 2011). Furthermore, limited emulsification and foaming properties of MPC compared to other milk proteins, such as WPC, WPI and sodium caseinate, can limit their use in processed meats, coffee creamer, whipped toppings and soups (Singh, 2011).

Functional properties of proteins are related to their molecular hierarchical structure composed of the primary and at least the secondary structure, which govern protein ability to interact with other components of a food matrix (Severin & Xia, 2006). Therefore, efficient utilization of milk proteins in food systems depends on tailoring their structural characteristics. Extensive hydrolysis, however, is not feasible approach as it leads to many properties of

Table 4
Selected milk protein hydrolysates obtained by plant proteases (either as a single step hydrolysis or with other group of proteases) to evaluate reduction in antigenicity and allergenicity.

Enzyme	Substrate	Hydrolysis conditions	Immunogenicity reduction	Reference
Alcalase, papain	WPC	15 mAU g^{-1} protein/50 °C/pH 8/120 min (single step) and 100 min for 1st enzyme followed by 20 min for 2nd enzyme (two steps)	Two steps hydrolysis was more effective in reducing antigenicity but allergenic epitopes were still present	Wróblewska et al. (2004)
Four combinations of trypsin, neutrase, papain, protease S	Freeze dried demineralized cheese whey	E/S ratio 1:100, pH 8.0 at 50 °C/180 min	Trypsin/papain (1/1) and Trypsin/neutrase (1/1) showed about 40% reduction in antigenicity	Shin et al. (2007)
Pronase, Papain, corolase 7089, alcalase, neutrase Chymotrypsin	WPC (78% protein)	E/S (1/25) 40 °C for all enzymes except alcalase, neutrase and corolase (50 °C) for 5 min under microwave treatment (MWI)	Significant decrease in immunoreactivity was observed in hydrolysates obtained by combining MWI and Pronase, Papain or Alcalase	Izquierdo et al. (2008)
Alcalase and Bromelain	β-LG	3% β-LG (w/v), 25 U enzyme g^{-1} of protein, pH 7.5, and temperature of 60 and 55 °C for alcalase and bromelain, respectively followed by TGase polymerization of hydrolysates	Hydrolysis associated or not with polymerization reduced the number of epitopes and the IgE-binding capacity of native β-LG	Villas-Boas et al. (2015)
Actinidin	WPI and MPC	E:S = 1:100; uncontrolled pH; 15 °C (31 h) and 60 °C (5 h)	At 60 °C, antigenicity reduction for MPC = β-LG (43%) & αs_1-CN (48%). WPI = β-LG (54%). At 10 °C, antigenicity reduction for MPC = β-LG (39%) & αs_1-CN (42%). WPI = β-LG (14%).	Kaur et al. (2022)
Latex peptidase (CpLP) *Calotropis procera*	Caseins	E/S 1:75; pH 6.5; 37 °C; 30 min	Residual antigenicity % of control = 100%; CpLP = 2%; CgLP = 1%; CapLP = 2%	Oliveira et al. (2019)
Latex peptidase (CgLP) *Cryptostegia grandiflora*	Whey proteins	E/S 1:75; pH 6.5; 37 °C; 24 h	Residual antigenicity % of control = 100%; CpLP = 78%; CgLP = 71%; CapLP = 31%	Oliveira et al. (2019)
Latex peptidase (PrLP) *Plumeria rubra*				
papain	Cow milk	E/S 2000–10,000 U g^{-1}; 20 °C; 120 min	%DH = around 2.0–4.5%; IgG reactivity reduction = approx. 75%	Liang et al. (2020)
Serine protease from *Cucurbita ficifolia*	WPC	E/S 150 U mg^{-1}; 37 °C; time range = 1–24 h	%DH range = around 19–44%; IgG reactivity reduction = approx. 60%; IgE reactivity reduction = approx. 23%	Babij et al. (2015)
	αs-casein		%DH range = around 34–61%; 1 h hydrolysis (34% DH) significantly reduced antigenicity.	

these hydrolysates limiting their usage in products such as milk formulae due to bitter taste, off flavour, increased osmolality and low emulsifying ability (Foegeding, Davis, Doucet, & McGuffey, 2002). Thus, functional properties of milk proteins may be improved by limited proteolysis.

Several studies have investigated the effectiveness of plant proteases in hydrolysing dairy proteins. Table 5 shows some of the applications of selected plant proteases in dairy to evaluate hydrolysis and functional properties. Plant proteases are also compared with proteases from other sources, on functional properties of dairy proteins and the outcomes showed appreciable effect of plant-based enzymes over others. For example, Luo, Pan, and Zhong (2014) used papain, pancreatin and trypsin for hydrolysing sodium caseinate and reported that caseinate treated with papain exhibited highest solubility with increased degree of hydrolysis, as compared to pancreatin and trypsin. Furthermore, studies on the hydrolysis of MPC with showed significant reductions in insolubility for treatment with papain, trypsin and chymotrypsin as compared to pepsin (Banach, Lin, & Lamsal, 2013). On evaluating emulsifying and foaming properties of camel milk proteins treated with alcalase, bromelain or papain, camel milk proteins treated with papain showed the highest foaming capacity and emulsifying activity, as compared to those treated with alcalase and bromelain (Al-Shamsi, Mudgil, Hassan, & Maqsood, 2018).

It has been observed that at a high degree of hydrolysis, solubility can be increased, and viscosity can be decreased (Abd-El-Salam, El-Shibiny, & Salem, 2009). Similarly, Banach et al. (2013) also observed an increase in solubility of MPC after proteolytic hydrolysis with papain, with the control showing around 45% solubility at pH 7 and solubility increased to 70 and 78% after papain hydrolysis for 30 and 180 min, respectively. Caseins and sodium caseinate have shown improved solubility at isoelectric point (Sitohy, Chobert, & Haertlé, 2001). A study of MPC and WPC hydrolysates obtained by treatment with actinidin also showed improved functional properties, such as foaming and solubility improved for both substrates, where whey proteins hydrolysates attained more than 97% solubility (Kaur, Vasiljevic, & Huppertz, 2023a). In contrast, both hydrolysed substrates showed worse emulsifying properties than intact proteins (Kaur et al., 2023a). However, very limited information is available on the heat stability comparisons of reconstituted MPC powders with plant based proteolytic hydrolysis treatment.

3.3. Reducing bitterness of protein hydrolysates

While some applications of proteases have been successful to produce hydrolysates with reduced allergenicity and improved solubility, problems associated with poor taste of completely hydrolysed proteins remain. During enzymatic hydrolysis in the initial stage, larger polypeptides are generated that mostly larger than 6 kDa. Due to complexity of molecular structure, these peptides are unable to reach taste receptors, thus do not impart bitterness. As degree of hydrolysis progresses, rapid decrease of molecular weight of peptides (under 6 kDa) generates more short chain hydrophobic peptides that increases hydrophobicity of solution/product. Ney (1971) showed that peptides (<6 kDa) containing higher content of Leu, Pro, Phe, Tyr, Ile and Trp residues are bitter. Overall hydrolysates obtained by either limited or extensive hydrolysis, polypeptide chains containing higher amount of hydrophobic amino acids would impart bitterness, whereas peptides

Table 5
Selected plant proteases in dairy products taken to evaluate % DH and improved functional properties.

Substrate type	Enzyme used	Parameters	% DH	Control measurements	Functionality Improvement	References
Sodium caseinate	Papain	pH 7/37 °C/10 min to 24 h/Enzyme to substrate ratio 0.5:100	13.32%–22.06%	EAI of unhydrolyzed sample – 175.64 $m^2 g^{-1}$	EAI – 383.53 $m^2 g^{-1}$ (highest at 10 min of incubation)	Luo et al. (2014)
				ESI of unhydrolyzed sample – 33.79 min	ESI – 93.42 min (highest at 10 min of incubation)	
		pH 4–9		Solubility 10%–90% (dependent of pH)	Above 80%–90% (at all pH levels)	
Camel milk	Papain	pH 7/50 °C/6 h/Enzyme to substrate ratio 1:100	39.6%	EAI of unhydrolyzed sample – 55.361 $m^2 g^{-1}$	EAI – 86.135 $m^2 g^{-1}$	Al-Shamsi et al. (2018)
	Bromelain		23.8%		EAI – 55.361 $m^2 g^{-1}$	
MPC	Papain	pH 6.8/60.0 °C/30–180 min	7.2–9.8%	Emulsion Activity	Emulsion Activity unchanged after hydrolysis	Banach et al. (2013)
				Foaming capacity (mL mL^{-1}) for control 1.71	After hydrolysis 1.68 to 1.70 (mL mL^{-1})	
		pH 6/40 °C/30–120 min		Protein solubility 7.4% (control)	Protein solubility was 12.6% after hydrolysis	
MPC	Actinidin	pH 7/60.0 °C/5 h	DH% – 0, 5, 10 and 15%	Solubility – approx. 50%; heat stability 90.7%; foam overrun 344%; foam stability 1260 s. (control)	Solubility – approx. 65%; heat stability 95.4%; foam overrun 406%; foam stability 2454 s.	Kaur et al. (2023a)
WPC				Solubility – approx. 83%; heat stability 71%; foam overrun 0%; foam stability 0 s.	Solubility – approx. 97%; heat stability 95%; foam overrun 270%; foam stability 120 s.	
WPC	Ficin	pH 7.5/80.0 °C/0.5–6 h	DH% – around 18–38%	Solubility – 48% (at pH 5) and 65% (at pH 7)	Solubility – 98% (at pH 5) and 85% (at pH 7)	Kheroufi, Brassesco, Campos, Boughellout, and Pintado (2022)
WPC	Prolyve	pH 7.0/50.0 °C/1 and 4 h	DH% – around 7%	Apparent viscosity – around 2 mPa s (for 1–4 h incubation)	Apparent viscosity = <2 mPa s (for both 1 and 4 h incubation)	Gruppi, Dermiki, Spigno, and FitzGerald (2022)
MPC			DH% – around 8%	Apparent viscosity – around 2 mPa s (for 1–4 h incubation)	Apparent viscosity – around 1.5–2.5 mPa s (1–4 h incubation)	
Sodium caseinate			DH% – around 10%	Apparent viscosity – around 5 mPa s (1–4 h incubation)	Apparent viscosity – around 2 mPa s (1–4 h incubation)	

with fewer or no hydrophobic amino acids would yield bitterness that would be negligible (Liu et al., 2022). Therefore, after controlled hydrolysis with plant proteases, careful selection and separation of peptide chains containing only hydrophilic amino acids would also be a viable solution to bitterness.

Trp, Ile, Tyr, Phe, Pro, Leu, and Val are amino acids that can contribute to bitterness. For example, free Leu or Phe present are bitter, but bitterness increases around 10-fold further when they are present as Leu-Phe, Leu-Leu or Ile-Leu (Kim & Li, 2006). Also, a presence of Pro amino acid in the middle of some peptides renders strong bitterness (Ishibashi et al., 1988). Furthermore, the presence of Arg next to Pro can enhances bitterness further. However, Gly is neutral and prevents bitterness when placed in between Pro and Arg (Ishibashi et al., 1988). It is evident from many studies that bitterness stems from hydrophobic amino acids present at the peptide termini (Bouchier, O'cuinn, Harrington, & Fitzgerald, 2001; Edens et al., 2005; Izawa, Tokuyasu, & Hayashi, 1997; Nishiwaki, Yoshimizu, Furuta, & Hayashi, 2002). Therefore, if generated peptides have hydrophobic amino acids not at the terminal end of peptide chains it can reduce bitterness. Also, a study conducted by Izawa et al. (1997) showed that hydrolysates generated by D3 contains hydrophobic amino acids that were mostly not present on the peptide terminals and thus resulted in less bitterness. In addition, a study conducted by Matoba and Hata (1972) showed that hydrophobic amino acids present on the carboxy- or amino-end of the peptides are more bitter as compared to these amino acids scattered in the middle of the peptide chain.

As stated in previous sections, plant proteases have promising approach to be used in control hydrolysis and may combat bitterness. For example, treatment with plant protease D3, obtained from soybean cotyledons, yielded less bitter casein hydrolysates compared to those prepared with trypsin, pepsin and subtilisin (Izawa et al., 1997). Another study by Wróblewska et al. (2004) showed that papain rendered fewer bitter peptides of WPC hydrolysates as compared to pepsin or alcalase. Also, in another study, 3 h of hydrolysis of casein with a commercial plant protease Promod 523MDP ™ (bromelain) resulted in significant reduction of bitterness (Daher et al., 2021). Many studies have reported that plant proteases, such as cathepsin L, cathepsin K and D3, prefer hydrophobic amino acids at position P2 of specific substrate to act on (Asano, Suzuki, Kawai, Miwa, & Shibai, 1999; Kirschke, Barrett, & Rawlings, 1995; McQueney et al., 1997). As we can see from previous section that papain, bromelain, ficin and actinidin also prefer hydrophobic amino acid at P2 position to act on, thus there is a greater possibility that all the above indicated proteases would act similarly to combat bitterness.

3.4. Release of bioactive peptides

Bioactive peptides can be released from milk proteins by enzymatic hydrolysis. These peptides can play an important role in nutrition, immune system (antimicrobial peptides and immunomodulating peptides), nervous system (opioid peptides) and cardiovascular system (antihypertensive peptides and antithrombotic peptides) (Silva & Malcata, 2005). Many studies have been done on milk proteins by using plant proteases to obtain bioactive peptides (Chew, Toh, & Ismail, 2019; Mazorra-Manzano, Ramírez-Suarez, & Yada, 2018; Mudgil et al., 2019).

Angiotensin converting enzyme (ACE) inhibitory peptides have the ability to lower blood pressure by limiting the vasoconstriction of angiotensin II. In a study where papain, pancreatin or trypsin were used to hydrolyse sodium caseinate, ACE-inhibitory activities after use of papain were significantly higher (about 70%) compared to trypsin (about 65%) and pancreatin (about 40%) (Luo et al., 2014). Also, bromelain (E:S = 1:100) was used with half skimmed and UHT milk and resulted in significant increase in ACE inhibitory activity of about 36% and 44%, respectively (Medeiros, Rainha, Paiva, Lima, & Baptista, 2013). Alcalase, papain and bromelain were also used

with camel milk proteins to compare their efficiency, and papain and bromelain showed significantly higher ACE inhibition as compared to alcalase (Mudgil et al., 2019).

Also in another study (Al-Shamsi et al., 2018) peptides (<14 kDa) of camel milk hydrolysate obtained by papain and bromelain showed significant improvement in antioxidant activity as compared to their controls. For example, DPPH (2,2-diphenyl-1-picrylhydrazyl) activity increased by 50% and 33%, ABTS (2,2-azinobis 3-ethylbenzthiazoline-6-sulfonic acid) radical scavenging activity increased by around 5 and 12 times, and ferrous Iron-Chelating activity 21% and 2%, for bromelain and papain. Another study by Luo et al. (2014) also indicated that casein hydrolysis by papain (at about 22% DH) showed significant improvement in DPPH scavenging activity (increased by 50%), and in ACE inhibitory activity (increased by 9 times) as compared to control.

Higher oxygen radical absorbance capacity (ORAC) value was obtained after whey proteins were hydrolysed by papain at neutral pH. Whey hydrolysates obtained by treatment with papain also displayed higher DPP-IV (dipeptidyl peptidase) inhibitory activities as compared to hydrolysis at controlled pH. These bioactive changes appeared to be due to pH changes, which resulted in different enzymatic conformations (Le Maux, Nongonierma, Barre, & Fitzgerald, 2016). Kumar, Chatli, Singh, Mehta, and Kumar (2016) reported a significantly higher ABTS radical scavenging activity of camel milk protein hydrolysates by papain after 6 h of process.

DPP-IV (dipeptidyl peptidase-IV) is an enzyme involved in glucose homeostasis and can results in malfunction of endocrine, immune and inflammatory system (Abd-El-Salam et al., 2009). Preparing hydrolysates of peptides enriched with DPP-IV inhibitory activity such as type 2 diabetes mellitus, immunological disorders and obesity, are of great interest. A study conducted by Boots (2013) on casein hydrolysis with number of enzymes including plant proteases, showed that permeate obtained after hydrolysates fractionation exhibited significant DPP-IV inhibitory activity (peptide contained minimum 1 proline residue at N-terminal).

4. Conclusions and future perspectives

Plant proteases are extremely versatile with diverse specificities and applications. As discussed so far, numerous studies often conducted on enzymatic hydrolysis of proteins, problems such as taste in extensively hydrolysed proteins (while maintaining its nutritional value) and poor stability in partially hydrolysed proteins (need better understanding of structural characteristics and interactions of hydrolysates), reduced allergenicity (by having better understanding of specific epitopes) and functional properties still require further clarification. Hence, understanding of characterisation of plant-based enzymes have potential to resolve issues with milk proteins addressed in literature review above and evaluate allergenicity, digestibility and functional properties of milk proteins.

CRediT authorship contribution statement

S. Kaur: Writing – original draft, Conceptualization. **T. Huppertz:** Writing – review & editing, Supervision, Conceptualization. **T. Vasiljevic:** Writing – review & editing, Supervision, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

Abd-El-Salam, M. H., El-Shibiny, S., & Salem, A. (2009). Factors affecting the functional properties of whey protein products: A review. *Food Reviews International, 25*, 251–270.

Abe, N., Wu, C. Y., Kim, Y. K., Fujii, T., & Abe, K. (2015). Development of an efficient soymilk cream production method by papain digestion, heat treatment, and low-speed centrifugation. *Bioscience, Biotechnology, and Biochemistry, 11*, 1890–1892.

Ahmed, I. A. M., Morishima, I., Babiker, E. E., & Mori, N. (2009). Dubiumin, a chymotrypsin-like serine protease from the seeds of Solanum dubium Fresen. *Phytochemistry, 70*, 483–491.

Aider, M. (2021). Potential applications of ficin in the production of traditional cheeses and protein hydrolysates. *JDS Communications, 2*, 233–237.

Al-Shamsi, K. A., Mudgil, P., Hassan, H. M., & Maqsood, S. (2018). Camel milk protein hydrolysates with improved technofunctional properties and enhanced antioxidant potential in in vitro and in food model systems. *Journal of Dairy Science, 101*, 47–60.

Anandan, R., Sudhakar, D., Balasubramanian, P., & Gutiérrez-Mora, A. (2012). In vitro somatic embryogenesis from suspension cultures of Carica papaya L. *Scientia Horticulturae, 136*, 43–49.

Arshad, Z. I. M., Amid, A., Yusof, F., Jaswir, I., Ahmad, K., & Loke, S. P. (2014). Bromelain: An overview of industrial application and purification strategies. *Applied Microbiology and Biotechnology, 98*, 7283–7297.

Asano, M., Suzuki, S., Kawai, M., Miwa, T., & Shibai, H. (1999). Characterization of novel cysteine proteases from germinating cotyledons of soybean [*Glycine max* (L.) Merrill]. *Journal of Biochemistry, 126*, 269–301.

Asif-Ullah, M., Kim, K.-S., & Yu, Y. G. (2006). Purification and characterization of a serine protease from *Cucumis trigonus* Roxburgui. *Phytochemistry, 67*, 870–875.

Babij, K., Bajzert, J., Dąbrowska, A., Szołtysik, M., Zambrowicz, A., Lubec, G., et al. (2015). Hydrolysis with Cucurbita ficifolia serine protease reduces antigenic response to bovine whey protein concentrate and αs-casein. *Amino Acids, 47*, 2335–2343.

Baker, E. N., Boland, M. J., Calder, P. C., & Hardman, M. J. (1980). The specificity of actinidin and its relationship to the structure of the enzyme. *Biochimica et Biophysica Acta (BBA) - Enzymology, 616*, 30–34.

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.

Bey, N., Debbebi, H., Abidi, F., Marzouki, M. N., & Salah, A. B. (2018). The non-edible parts of fennel (Fœniculum vulgare) as a new milk-clotting protease source. *Industrial Crops and Products, 112*, 181–187.

Bogahawaththa, D., Chandrapala, J., & Vasiljevic, T. (2017). Modulation of milk immunogenicity by thermal processing. *International Dairy Journal, 69*, 23–32.

Boland, M., & Singh, J. (2013). Chapter 429-Actinidain. In N. D. Rawlings, & G. Salvesen (Eds.), *Handbook of proteolytic enzymes* (3rd ed., pp. 1879–1884). Cambridge, Massachusetts, USA: Academic Press.

Boots, J. W. P. (2013). Protein hydrolysate enriched in peptides inhibiting DPP-IV and the use. US Patent 20130096074.

Bouchier, P. J., O'Cuinn, G., Harrington, D., & Fitzgerald, R. J. (2001). Debittering and hydrolysis of a tryptic hydrolysate of β-casein with purified general and praline specific aminopeptidase from *Lactococcus lactis* ssp. cremoris AM2. *Journal of Food Science, 66*, 816–820.

Brutti, C. B., Pardo, M. F., Caffini, N. O., & Natalucci, C. L. (2012). Onopordum acanthium L.(Asteraceae) flowers as coagulating agent for cheesemaking. *LWT - Food Science and Technology, 45*, 172–179.

Carne, A., & Moore, C. H. (1978). The amino acid sequence of the tryptic peptides from actinidin, a proteolytic enzyme from the fruit of *Actinidia chinensis*. *Biochemical Journal, 173*, 73–83.

Chatchatee, P., Jarvinen, K. M., Bardina, L., Vila, L., Beyer, K., & Sampson, H. A. (2001). Identification of IgE and IgG binding epitopes on β- and κ-casein in cow's milk allergic patients. *Clinical and Experimental Allergy, 31*, 1256–1262.

Chew, L. Y., Toh, G. T., & Ismail, A. (2019). Application of proteases for the production of bioactive peptides. In M. Kuddus (Ed.), *Enzymes in food biotechnology: Production, applications, and future prospects* (pp. 247–261). London, UK: Academic Press.

Cimino, C., Cavalli, S. V., Spina, F., Natalucci, C., & Priolo, N. (2006). Callus culture for biomass production of milk thistle as a potential source of milk-clotting peptidases. *Journal of Biotechnology, 9*, 237–240.

Cimino, C. V., Colombo, M. L., Liggieri, C., Bruno, M., & Vairo-Cavalli, S. (2015). Partial molecular characterization of Arctium minus aspartylendopeptidase and preparation of bioactive peptides by whey protein hydrolysis. *Journal of Medicinal Food, 18*, 856–864.

Cong, Y., Yi, H., Qing, Y., & Li, L. (2013). Identification of the critical amino acid residues of immunoglobulin E and immunoglobulin G epitopes on αs1-casein by alanine scanning analysis. *Journal of Dairy Science, 96*(11), 6870–6876.

Daher, D., Deracinois, B., Courcoux, P., Baniel, A., Chollet, S., Froidevaux, R., et al. (2021). Sensopeptidomic kinetic approach combined with decision trees and random forests to study the bitterness during enzymatic hydrolysis kinetics of micellar caseins. *Foods, 10*, 1312.

Devaraj, K. B., Gowda, L. R., & Prakash, V. (2008). An unusual thermostable aspartic protease from the latex of *Ficus racemosa* (L.). *Phytochemistry, 69*, 647–655.

Dini, I., Falanga, D., Di Lorenzo, R., Tito, A., Carotenuto, G., Zappelli, C., et al. (2021). An extract from Ficus carica cell cultures works as an anti-stress ingredient for the skin. *Antioxidants, 10*, 515.

Dupont, D., Croguennec, T., Brodkorb, A., & Kouaouci, R. (2013). Quantitation of proteins in milk and milk products. In P. L. H. McSweeney, & P. F. Fox (Eds.), *Advanced dairy chemistry: Volume 1A: Proteins: Basic aspects* (4th ed., pp. 87–134). Boston, MA: Springer US.

Edens, L., Dekker, P., Hoeven, R. V. D., Deen, F., Orrs, A. D., & Floris, R. (2005). Extracellular prolyl endoprotease from *Aspergillus niger* and its use in the debittering of protein hydrolysates. *Journal of Agricultural and Food Chemistry, 53*, 7950–7957.

Elateeq, A. A., Sun, Y., Nxumalo, W., & Gabr, A. M. (2020). Biotechnological production of silymarin in *Silybum marianum* L.: A review. *Biocatalysis and Agricultural Biotechnology, 29*, Article 101775.

Exl, B. M., & Fritsche, R. (2001). Cow's milk protein allergy and possible means for its prevention. *Nutrition, 17*, 642–651.

Feijoo-Siota, L., & Villa, T. G. (2011). Native and biotechnologically engineered plant proteases with industrial applications. *Food and Bioprocess Technology, 4*, 1066–1088.

Fernández-Lucas, J., Castañeda, D., & Hormigo, D. (2017). New trends for a classical enzyme: Papain, a biotechnological success story in the food industry. *Trends in Food Science & Technology, 68*, 91–101.

Fernandez, G., & Pomilio, A. B. (2003). Optimized growth conditions and determination of the catalytic type of the peptidase complex from a novel callus culture of pineapple (*Ananas comosus*). *Molecular Biology in Medicinal Chemistry, 1*, 39–49.

Foegeding, E. A., Davis, J. P., Doucet, D., & McGuffey, M. K. (2002). Advances in modifying and understanding whey protein functionality. *Trends in Food Science & Technology, 13*, 151.

Folgado, A., Pires, A. S., Figueiredo, A. C., Pimentel, C., & Abranches, R. (2020). Toward alternative sources of milk coagulants for cheese manufacturing: Establishment of hairy roots culture and protease characterization from *Cynara cardunculus* L. *Plant Cell Reports, 39*, 89–100.

Fox, P. F. (2003). Milk proteins, general and historical aspects, 319–386. In P. F. Fox, & P. L. H. McSweeney (Eds.), *Advanced dairy chemistry-proteins: Part A* (3rd ed., pp. 1–49). New York, NY, USA: Kluwer Academic, Plenum Publishers.

Freidl, R., Garib, V., Linhart, B., Haberl, E. M., Mader, I., Szépfalusi, Z., et al. (2022). Extensively hydrolyzed hypoallergenic infant formula with retained t cell reactivity. *Nutrients, 15*, 111.

Fritsché, R. (1998). Induction of oral tolerance to cow's milk proteins in rats fed with a whey protein hydrolysate. *Nutrition Research, 18*, 1335–1341.

Gavira, J. A., Gonzalez-Ramirez, L. A., Oliver-Salvador, M. C., Soriano-Garcia, M., & Garcia-Ruiz, J. M. (2007). Structure of the mexicain-E-64 complex and comparison with other cysteine proteases of the papain family. *Acta Crystallographica, 63*, 555–563.

Ghosh, B. C., Prasad, L. N., & Saha, N. P. (2017). Enzymatic hydrolysis of whey and its analysis. *Journal of Food Science and Technology, 54*, 1476–1483.

Grozdanović, M. M., Gavrović-Jankulović, M., & Drakulić, B. J. (2013). Conformational mobility of active and E-64-inhibited actinidin. *Biochimica et Biophysica Acta - General Subjects, 1830*, 4790–4799.

Grozdanovic, M. M., Burazer, L., & Gavrovic-Jankulovic, M. (2013). Kiwifruit (*Actinidia deliciosa*) extract shows potential as a low-cost and efficient milk-clotting agent. *International Dairy Journal, 32*, 46–52.

Gruppi, A., Dermiki, M., Spigno, G., & FitzGerald, R. J. (2022). Impact of enzymatic hydrolysis and heat inactivation on the physicochemical properties of milk protein hydrolysates. *Foods, 11*, 516.

Gupta, N., Jain, V., Joseph, M. R., & Devi, S. (2020). A review on micropropagation culture method. *Asian Journal of Pharmaceutical Research and Development, 8*, 86–93.

Gurumallesh, P., Alagu, K., Ramakrishnan, B., & Muthusamy, S. (2019). A systematic reconsideration on proteases. *International Journal of Biological Macromolecules, 128*, 254–267.

Hasegawa, A., Sogawa, K., Takahashi, Y., Satoh, M., Nomura, F., & Shimojo, N. (2017). Hypoallergenic cow's milk by bromelain. *Electrophoresis Letters, 61*, 35–38.

Havea, P. (2006). Protein interactions in milk protein concentrate powders. *International Dairy Journal, 16*, 415–422.

Host, A., & Halken, S. (2014). Cow's milk allergy: Where have we come from and where are we going? *Endocrine, Metabolic & Immune Disorders Drug Targets, 14*, 2–8.

Huppertz, T. (2013). The chemistry of caseins. In P. L. H. McSweeney, & P. F. Fox (Eds.), *Advanced dairy chemistry: Proteins* (4th ed., pp. 135–160). New York, US: Springer.

Ishibashi, N., Ono, I., Kato, K., Shigenaga, T., Shinoda, I., Okai, H., et al. (1988). Role of the hydrophobic amino acid residue in the bitterness of peptides. *Agricultural and Biological Chemistry, 52*, 91–94.

Izawa, N., Tokuyasu, K., & Hayashi, K. (1997). Debittering of protein hydrolysates using *Aeromonas caviae* aminopeptidase. *Journal of Agricultural and Food Chemistry, 45*, 543–545.

Izquierdo, F. J., Peñas, E., Baeza, M. L., & Gomez, R. (2008). Effects of combined microwave and enzymatic treatments on the hydrolysis and immunoreactivity of dairy whey proteins. *International Dairy Journal, 18*, 918–922.

Järvinen, K., Chatchatee, P., Bardina, L., Beyer, K., & Sampson, H. A. (2001). IgE and IgG binding epitopes on α-lactalbumin and β-lactoglobulin in cow's milk allergy. *International Archives of Allergy and Immunology, 126*, 111–118.

Kaur, S., Huppertz, T., & Vasiljevic, T. (2021). Milk protein hydrolysis by actinidin: Influence of protein source and hydrolysis conditions. *International Dairy Journal, 118*, Article 105029.

Kaur, S., Huppertz, T., & Vasiljevic, T. (2022). Actinidin-induced hydrolysis of milk proteins: Effect on antigenicity. *LWT - Food Science and Technology, 161*, Article 113294.

Kaur, S., Vasiljevic, T., & Huppertz, T. (2023a). Influence of actinidin-induced hydrolysis on the functional properties of milk protein and whey protein concentrates. *Foods, 12*, 3806.

Kaur, S., Vasiljevic, T., & Huppertz, T. (2023b). Milk protein hydrolysis by actinidin - Kinetic and thermodynamic characterisation and comparison to bromelain and papain. *Foods, 12*, 4248.

Kheroufi, A., Brassesco, M. E., Campos, D. A., Boughellout, H., & Pintado, M. E. (2022). Functional properties of peptides obtained from whey proteins by ficin extract hydrolysis. *Food Bioscience, 47*, Article 101707.

Kim, H. O., & Li-Chan, E. C. Y. (2006). Quantitative structure–activity relationship study of bitter peptides. *Journal of Agricultural and Food Chemistry, 54*(26), 10102–10111.

Kirschke, H., Barrett, A. J., & Rawlings, N. D. (1995). Lysosomal cysteine proteases. In P. Sheterline (Ed.), *Protein profile* (pp. 1587–1643). London, UK: Academic Press.

Krem, M. M., Rose, T., & Di Cera, E. (2000). Sequence determinants of function and evolution in serine proteases. *Trends in Cardiovascular Medicine, 10*, 171–176.

Kumar, D., Chatli, M. K., Singh, R., Mehta, N., & Kumar, P. (2016). Enzymatic hydrolysis of camel milk proteins and its antioxidant properties. *Journal of Camel Practice and Research, 23*, 33–40.

Kumari, M., Sharma, A., & Jagannadham, M. V. (2010). Decolorization of crude latex by activated charcoal, purification and physico-chemical characterization of religiosin, a milk-clotting serine protease from the latex of *Ficus religiosa*. *Journal of Agricultural and Food Chemistry, 58*, 8027–8034.

Laplaze, L., Ribeiro, A., Franche, C., Duhoux, E., Auguy, F., Bogusz, D., et al. (2000). Characterization of a Casuarina glauca nodule-specific subtilisin-like protease gene, a homolog of Alnus glutinosa ag12. *Molecular Plant-Microbe Interactions, 13*, 113–117.

Le Maux, S., Nongonierma, A. B., Barre, C., & FitzGerald, R. J. (2016). Enzymatic generation of whey protein hydrolysates under pH-controlled and non pH-controlled conditions: Impact on physicochemical and bioactive properties. *Food Chemistry, 199*, 246–251.

Liang, X., Yang, H., Sun, J., Cheng, J., Luo, X., Wang, Z., et al. (2020). Effects of enzymatic treatments on the hydrolysis and antigenicity reduction of natural cow milk. *Food Science and Nutrition, 9*, 985–993.

Liburdi, K., Spinelli, S. E., Benucci, I., Lombardelli, C., & Esti, M. (2018). A preliminary study of continuous milk coagulation using *Cynara cardunculus* flower extract and calf rennet immobilized on magnetic particles. *Food Chemistry, 239*, 157–164.

Liu, B., Li, N., Chen, F., Zhang, J., Sun, X., Xu, L., et al. (2022). Review on the release mechanism and debittering technology of bitter peptides from protein hydrolysates. *Comprehensive Reviews in Food Science and Food Safety, 21*, 5153–5170.

Lorenzo, J. M., Munekata, P. E. S., Gómez, B., Barba, F. J., Mora, L., Pérez-Santaescolástica, C., et al. (2018). Bioactive peptides as natural antioxidants in food products – A review. *Trends in Food Science & Technology, 79*, 136–147.

Luo, Y., Pan, K., & Zhong, Q. (2014). Physical, chemical and biochemical properties of casein hydrolyzed by three proteases: *Partial characterizations*. *Food Chemistry, 155*, 146–154.

Mahajan, R. T., & Chaudhari, G. M. (2014). Plant latex as vegetable source for milk clotting enzymes and their use in cheese preparation. *International Journal of Advanced Research, 5*, 1173–1181.

Matoba, T., & Hata, T. (1972). Relationship between bitterness of peptides and their chemical structure. *Agricultural and Biological Chemistry, 36*, 1423–1431.

Mazorra-Manzano, M. A., Moreno-Hernández, J. M., Ramírez-Suarez, J. C., de Jesús Torres-Llanez, M., González-Córdova, A. F., & Vallejo-Córdoba, B. (2013). Sour orange *Citrus aurantium* L. flowers: A new vegetable source of milk-clotting proteases. *LWT - Food Science and Technology, 54*, 325–330.

Mazorra-Manzano, M. A., Ramírez-Suarez, J. C., & Yada, R. Y. (2018). Plant proteases for bioactive peptides release: A review. *Critical Reviews in Food Science and Nutrition, 58*, 2147–2163.

McQueney, M. S., Amegadzie, B. Y., D'Alesssio, K., Hanning, C. R., McLaughlin, M. M., McNulty, D., et al. (1997). Autocatalytic activation of human cathepsin K. *Journal of Biological Chemistry, 272*, 13955–13960.

Medeiros, V., Rainha, N., Paiva, L., Lima, E., & Baptista, J. (2013). Bovine milk formula based on partial hydrolysis of caseins by bromelain enzyme: Better digestibility and angiotensin-converting enzyme-inhibitory properties. *International Journal of Food Properties, 17*, 806–817.

Morellon-Sterling, R., El-Siar, H., Tavano, O. L., Berenguer-Murcia, Á., & Fernández-Lafuente, R. (2020). Ficin: A protease extract with relevance in biotechnology and biocatalysis. *International Journal of Biological Macromolecules, 162*, 394–404.

Mudgil, P., Baby, B., Ngoh, Y. Y., Kamal, H., Vijayan, R., Gan, C.-Y., et al. (2019). Molecular binding mechanism and identification of novel antihypertensive and anti-inflammatory bioactive peptides from camel milk protein hydrolyzates. *LWT - Food Science and Technology, 112*, Article 108193.

Nadarajan, J., Esfandiari, A., Mathew, L., Divinagracia, J., Wiedow, C., & Morgan, E. (2023). Development, management and utilization of a kiwifruit (*Actinidia* spp.) *In vitro* collection: A New Zealand perspective. *Plants, 12*, 2009.

Ney, K. H. (1971). Prediction of bitterness of peptides from their amino acid composition. *Zeitschrift für Lebensmittel-Untersuchung und -Forschung, 147*, 64–68.

Nishiwaki, T., Yoshimizu, S., Furuta, M., & Hayashi, K. (2002). Debittering of enzymatic hydrolysates using an aminopeptidase from edible basidiomycete *Grifola frondosa*. *Journal of Bioscience and Bioengineering, 93*, 60–63.

Noman, A., Xu, Y., Al-Bukhaiti, W. Q., Abed, S. M., Ali, A. H., Ramadhan, A. H., et al. (2018). Influence of enzymatic hydrolysis conditions on the degree of hydrolysis and functional properties of protein hydrolysate obtained from Chinese sturgeon (*Acipenser sinensis*) by using papain enzyme. *Process Biochemistry, 67*, 19–28.

Oliveira, J. P. B., Candreva, A. M., Rizzo, G., Ramos, M. V., Oliveira, J. S., Oliveira, H. D., et al. (2019). Allergenicity reduction of cow's milk proteins using latex peptidases. *Food Chemistry, 284*, 245–253.

Ovissipour, M., Rasco, B., Shiroodi, S. G., Modanlow, M., Gholami, S., & Nemati, M. (2013). Antioxidant activity of protein hydrolysates from whole anchovy sprat (Clupeonella engrauliformis) prepared using endogenous enzymes and commercial proteases. *Journal of the Science of Food and Agriculture, 93*, 1718–1726.

Palmer, T. (2001). *Enzymes: Biochemistry, biotechnology and clinical chemistry*. Chichester, West Sussex, England: Horwood.

Panjaitan, S. B., Aziz, M. A., Rashid, A. A., & Saleh, N. M. (2007). In vitro plantlet regeneration from shoot tip of field-grown hermaphrodite papaya (Carica papaya L. cv. Eksotika). *International Journal of Agriculture and Biology, 9*, 827–832.

Pasqual, M., & Ferreira, E. A. (2007). Micropropagation of fig tree (*Ficus carica* L.). In S. M. Jain, & H. Haggman (Eds.), *Protocols for micropropagation of woody trees and fruits* (pp. 409–416). Dordrecht, the Netherlands: Springer.

Pecquet, S., Bovetto, L., Maynard, F., & Fritsché, R. (2000). Peptides obtained by tryptic hydrolysis of bovine β-lactoglobulin induce specific oral tolerance in mice. *Journal of Allergy and Clinical Immunology, 105*, 514–521.

Pickersgill, R. W., Harris, G. W., & Garman, E. (1992). Structure of monoclinic papain at 1.60-A resolution. *Acta Crystallographica Section B, 48*, 59–67.

Polaina, J., & MacCabe, A. P. (2007). *Industrial enzymes: Structure, function and applications*. New York: Springer.

Prado, M. J., Herrera, M. T., Vázquez, R. A., Romo, S., & González, M. V. (2005). Micropropagation of two selected male kiwifruit and analysis of genetic variation with AFLP markers. *HortScience, 40*, 740–746.

Rajesh, R., Nataraju, A., Gowda, C. R., Frey, B. M., Frey, F. J., & Vishwanath, B. S. (2006). Purification and characterization of a 34-kDa, heat stable glycoprotein from Synadenium grantii latex: Action on human fibrinogen and fibrin clot. *Biochimie, 88*, 1313–1322.

Raposo, S., & Domingos, A. (2008). Purification and characterization milk-clotting aspartic proteinases from *Centaurea calcitrapa* cell suspension cultures. *Process Biochemistry, 43*, 139–144.

Rawlings, N. D., Barrett, A. J., Woessner, J. F., Jr., & Salvesen, G. (2012). *Handbook of proteolytic enzymes*. San Diego, USA: Elsevier Science & Technology.

Sah, B. N. P., McAinch, A. J., & Vasiljevic, T. (2016). Modulation of bovine whey protein digestion in gastrointestinal tract. *International Dairy Journal, 62*, 10–18.

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.

Severin, S., & Xia, W. S. (2006). Enzymatic hydrolysis of whey proteins by two different proteases and their effect on the functional properties of resulting protein hydrolysates. *Journal of Food Biochemistry, 30*, 77–97.

Shin, H. S., Kim, S. B., Kang, S. C., Khan, M. A., Kim, H. S., Shin, H. J., et al. (2007). Production of low antigenic cheese whey protein hydrolysates using mixed proteolytic enzymes. *Journal of the Science of Food and Agriculture, 87*, 2055–2060.

Silva, S. V., & Malcata, F. X. (2005). Caseins as source of bioactive peptides. *International Dairy Journal, 15*, 1–15.

Singh, H. (2011). Milk protein products | Functional properties of milk proteins A2. In John W. Fuquay (Ed.), *Encyclopedia of dairy sciences* (2nd ed., pp. 887–893). San Diego: Academic Press.

Sitohy, M., Chobert, J. M., & Haertlé, T. (2001). Improvement of solubility and of emulsifying properties of milk proteins at acid pHs by esterification. *Die Nahrung, 45*, 87–93.

Sumantha, A., Larroche, C., & Pandey, A. (2006). Microbiology and industrial biotechnology of food–grade proteases: A perspective. *Food Technology and Biotechnology, 44*, 211–220.

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.

Torres, M. J., Trejo, S. A., Martin, M. I., Natalucci, C. L., Aviles, F. X., & Lopez, L. M. I. (2010). Purification and characterization of a cysteine endopeptidase from *Vasconcellea quercifolia* A. St.- Hil. latex displaying high substrate specificity. *Journal of Agricultural and Food Chemistry, 58*, 11027–11035.

Uchikoba, T., Hosoyamada, S., Onjyo, M., Arima, K., Yonezawa, H., & Kaneda, M. (2001). A serine endopeptidase from the fruits of *Melothria japonica* (Thunb.) maxim. *Phytochemistry, 57*, 1–5.

Villas-Boas, M. B., Benedé, S., de Lima Zollner, R., Netto, F. M., & Molina, E. (2015). Epitopes resistance to the simulated gastrointestinal digestion of β-lactoglobulin submitted to two-step enzymatic modification. *Food Research International, 72*, 191–197.

Wróblewska, B., Karamać, M., Amarowicz, R., Szymkiewicz, A., Troszyńska, A., & Kubicka, E. M. (2004). Immunoreactive properties of peptide fractions of cow whey milk proteins after enzymatic hydrolysis. *International Journal of Food Science and Technology, 39*, 839–850.

Wróblewska, B., & Troszyńska, A. (2005). Enzymatic hydrolysis of cows whey milk proteins in the aspect of their utilization for the production of hypoallergenic formulas. *Polish Journal of Food and Nutrition Sciences, 14*, 349–357.

Wu, J. H. (2017). Establishment of *in vitro* culture systems for breeding new types of kiwifruit and for Actinidia genomics studies. *VII International Symposium on Production and Establishment of Micropropagated Plants, 1224*, 195–202.

Yadav, S. C., Pande, M., & Jagannadham, M. V. (2006). Highly stable glycosylated serine protease from the medicinal plant Euphorbia milii. *Phytochemistry, 67*, 1414–1426.

Yadav, R. P., Patel, A. K., & Jagannadham, M. V. (2012). Neriifolin S, a dimeric serine protease from *Euphorbia neriifolia* Linn.: Purification and biochemical characterisation. *Food Chemistry*, 1296–1304.

Zadeh, M. T. (2017). *Application of proteolytic enzymes in suppressing immunoreactivity and deactivation of epitopes antigenicity in whey protein isolates* (Master of Science). Halifax, Nova Scotia: Dalhousie University.

Zhang, B., Sun, Q., Liu, H.-J., Li, S-Z., & Jiang, Z.-Q. (2017). Characterization of actinidin from Chinese kiwifruit cultivars and its applications in meat tenderization and production of angiotensin I-converting enzyme (ACE) inhibitory peptides. *LWT, 78*, 1–7.

Zhu, X., Kaur, L., & Boland, M. (2018). Thermal inactivation of actinidin as affected by meat matrix. *Meat Science, 145*, 238–244.