Application of Plant Proteases in Hydrolysis of Dairy Proteins

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Abstract

Milk proteins are highly valuable nutritional components required for the proper development of infants and toddlers and the maintenance of muscles in the elderly. In addition, functional properties of various commercial milk proteins are widely exploited by the food industry. However, their application in food systems is hindered by several important issues including cow's milk protein allergy (CMPA) and poor stability during processing among the most prevalent. Actinidin is an important plant protease enzyme that is widely used in dairy systems to improve the properties and processability of milk proteins. The main aim of this research was to establish conditions (temperature, pH, enzyme to substrate ratio, time) required for optimal enzymatic performance on partially or completely hydrolysed reconstituted milk protein preparations to achieve enhanced hypoallergenicity or functionality. Partial hydrolysis, which involves proteases with very high specificity and the cleavage of a limited number of specific peptide bonds in the protein to yield a slightly modified form of the protein. In case of complete hydrolysis, which results in a more extensive hydrolysis of a protein including hydrolysis of multiple peptide bonds and eventually complete conversion into amino acids. Here enhanced hypoallergenicity relates to breakdown of epitope regions into non allergic small peptides or free amino acids. This tailoring of structural characteristics of milk proteins can also lead to improvement in functionality. Thus, allergenicity and functional properties (solubility, heat stability, foaming and emulsification ability) of milk proteins were also assessed as a function of the degree of hydrolysis.

The activity of any enzyme is influenced by several important factors including substrate concentration, pH, ionic strength and environment, and temperature. All of these factors play a role in maintaining or disturbing the conformation of enzymes and thus may either stimulate or inhibit enzyme activity. Variations in environmental pH or ionic quality may alter

electrostatic interactions among charged amino acid segments and induce conformational changes in the structure of the enzyme. Similarly, the enzyme conformation is held by weak forces which may be perturbed by temperature. Experiments were performed to establish conditions for the optimum hydrolytic activity of commercial proteases (actinidin, bromelain and papain) using milk protein preparations as a substrate. The optimum temperature for activity of plant proteases was determined by executing the enzymatic reaction at different temperatures (15–55 °C) using milk protein concentrate (MPC), whey protein concentrate (WPC) and whey protein isolate (WPI) preparations. Protein solutions were prepared at a constant concentration (5% w/w) by dispersing powder in simulated milk ultrafiltrate (SMUF) followed by continuous overnight mixing for complete hydration. The protease assay mixture at pH 6.8 and at a constant enzyme to substrate (E:S) ratio was incubated at various temperatures and the extent of cleavage of peptide bonds was determined using a spectrophotometric assay using trinitrobenzenesulfonic acid (TNBS). The optimum temperature is defined as the one resulting in the maximum of the degree of hydrolysis under experimental conditions. This study was also performed without pH control to assess the impact of pH change. Actinidin at an E:S ratio of 1:100 resulted in a greater degree of hydrolysis (%DH) of whey proteins. Altering the ratio did not result in substantial change of %DH of MPC. For all three enzymes (actinidin, bromelain and papain), cleavages of proteins were clearly time dependant ($p<0.05$) while pH, although not controlled, did not change significantly ($p>0.05$) during the incubation process. The %DH increased with increasing temperature and the maximum %DH was achieved at 60 °C for all three dairy systems. PAGE analysis revealed that actinidin and papain mainly acted on α-lactalbumin and α_s-casein in WPI and MPC, respectively.

After following these protocols, hydrolysed samples were further assessed for antigenicity by the enzyme-linked immunosorbent assay (ELISA). For this, WPI and MPC substrates were

used with the aim to reduce immunoreactivity of hydrolysates of β-lactoglobulin (β-LG) and αs_1-casein (αs_1-CN) fractions of protein mixtures at 10 and 60 °C when treated with actinidin. Firstly, the %DH was determined by TNBS at an enzyme to substrate ratio of 1:100 (5.21 units of actinidin activity g^{-1} of protein) at 10 and 60 °C for up to 31 and 5 hours, respectively, for both substrates at uncontrolled pH. The antigenicity was tested using ELISA which confirmed a significant reduction of antigenicity of β-LG and αs_1-CN with higher %DH by actinidin, possibly by fragmentation and masking of epitopes. At 60 °C, hydrolysis resulted in a reduction in antigenicity of about 43 and 48% for MPC in the case of β-LG and αs_1-CN, respectively, and approximately 54% for WPI (β-LG). Hydrolysates obtained at 10 °C also resulted in a reduction in antigenicity for MPC of β-LG and αs_1-CN by about 39 and 42% respectively, but only 14% for WPI (β-LG). Overall, it can be suggested that proteolysis by actinidin can reduce the antigenicity by modification of protein conformation, and cleavage and masking of conformational and linear epitopes of β-LG and αs_1-CN to a certain extent in milk protein systems.

The impact of selected parameters of milk protein hydrolysates (MPH) of MPC and WPC were assessed to explore the effect on the functional properties. Here 0, 5, 10 and 15% DH was achieved for each substrate which were then reacted with actinidin and evaluated using the TNBS assay. The results revealed that significant changes in the functionality of MPH are associated with %DH. The solubility of MPH increased with an increase in %DH whereas whey proteins attained more than 97% solubility. The PAGE analysis revealed that the most soluble proteins were α-lactalbumin and κ-casein in WPC and MPC respectively, and were therefore more susceptible to the enzymatic action of actinidin. Emulsifying properties showed a decreasing trend with increasing %DH whereas heat stability increased, and the foaming properties of both MPH substrates were improved. These results were further validated using FTIR spectroscopy and zeta potential, however, particle size showed a mixed trend.

Actinidin efficiency (kinetic and thermodynamic characteristics) was then compared with other proteases from the same CA1 family including papain and bromelain. The kinetic parameters (K_m, k_{cat}, V_{max}) of the proteases were assessed from a Lineweaver–Burk plot, by performing activity assays at different concentrations of substrates at concentrations of 20, 40, 60, 80 and 100 mg mL^{-1}. Incubations were performed at 60°C and reactions were triggered by adding 2.6 units of enzyme activity equivalents of actinidin or bromelain or papain to the samples. WPC hydrolysis was characterised with lower K_m, higher k_{cat} and higher V_{max} as compared to MPC in case of all three enzymes. The values of k_{cat} and K_m were used to determine the substrate turnover and binding affinity of each protease. The thermodynamic parameters of these enzymes with MPC and WPC were also determined over a temperature range of 15–60 °C and the results were favourable for the potential application of papain and actinidin in dairy formulations.

Overall, actinidin exerted an appreciable and specific enzymatic activity towards the dairy protein substrates tested. Its application was further assessed in milk proteins where limited hydrolysis resulted in modulation of specific functionalities and/or allergenicity. Further manipulation of hydrolysis parameters, processing conditions and pH control could be a promising approach to improving the solubility and further functionality of MPH formulations.

Declaration

"I, Surjit Kaur, declare that the PhD thesis entitled **Application of Plant Proteases in Hydrolysis of Dairy Proteins** is no more than 80,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

"I have conducted my research in alignment with the Australian Code for the Responsible Conduct of Research and Victoria University's Higher Degree by Research Policy and Procedures".

Signature: 

Date: 07/02/2024

Dedicated to the eternal Lord Guru Granth Sahib Ji

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

4. Kaur, S., Vasiljevic, T., & Huppertz, T. (2023). Milk Protein Hydrolysis by Actinidin—Kinetic and Thermodynamic Characterisation and Comparison to Bromelain and Papain. *Foods,* 12, 4248.

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List of Abbreviations and Units

ALA	alpha-lactalbumin
Ala	alanine
Arg	arginine
Asn	asparagine
Asp	aspartic acid
BLG	beta-lactoglobulin
BSA	bovine serum albumin
˚C	degree Celsius
CMPA	cow's milk protein allergy
CNS	caseins
Cys	cysteine
DH	degree of hydrolysis
ELISA	enzyme-linked immunosorbent assay
FTIR	Fourier transform infrared spectroscopy
g	gram
Glu	glutamic acid
Gln	glutamine
Gly	glycine
H_2SO_4	sulphuric acid
h	hour
HCl	hydrochloric acid
His	histidine
Hz	hertz

Ig	immunoglobulin
Ile	isoleucine
kDa	kilo Dalton
L	litre
Leu	leucine
LF	lactoferrin
Lys	lysine
mA	milliampere
Met	methionine
min	minute
mL	millilitre
mM	millimolar
MPC	milk protein concentrate
MPH	milk protein hydrolysates
MW	molecular weight
ng	nanogram
OPA	ortho phthalaldehyde
PAGE	polyacrylamide gel electrophoresis
pH	hydrogen ion concentration
Phe	phenylalanine
Pro	proline
RI	refractive index
rpm	revolution per minute
s	second
SAS	statistical analysis software

SDS	sodium dodecyl sulphate
SEM	standard error of the mean
Ser	serine
SH	thiol group
SS	disulphide
Thr	threonine
TMB	3,3,5,5-tetramethylbenzidine
TNBS	trinitrobenzenesulfonic acid
Trp	tryptophan
Tyr	tyrosine
UV	ultraviolet
V	volts
Val	valine
v/v	volume per volume
w/w	weight per weight
W	Watt
WPC	whey protein concentrates
WPI	whey protein isolates
α	alpha
β	beta
κ	kappa
μ	micro
μg	microgram
μL	microliter

Chapter 1

Introduction

1.1 Introduction

Milk proteins are a diverse group of proteins composed of caseins and whey proteins. Although caseins are nutritionally very valuable, their main role in food applications is to build and stabilise structures in various products. They are a versatile group themselves consisting of four major types named α_{s1}, α_{s2}, β and κ-caseins. On the other hand, whey proteins are considered the most nutritionally valuable proteins due to the abundance of branched chain amino acids (BCAA), which play a crucial role in the human diet by providing these important amino acids for muscle physiology (Sah, McAinch, & Vasiljevic, 2016). Whey proteins are also a very diverse group of proteins including α-lactalbumin, β-lactoglobulin, bovine serum albumin, lactoferrin, lactoperoxidase, and various immunoglobulins and enzymes. In addition to muscular health, whey protein derived peptides possess various beneficial physiologically important properties impacting the immune, cardiovascular, digestive and nervous systems. In addition, these proteins can be used in many non-food applications such as drying aids, pharmaceuticals (direct and excipients), films, and coatings (Ghosh, Prasad, & Saha, 2017).

Food allergies have been recognised as the sixth of the contemporary health problems worldwide (Li, Zhu, Zhou, & Peng, 2012). For humans, one of the first encountered food allergies is associated with cow's milk as infants and toddlers are usually first exposed to this food early in their lives (Høst, 2002). The cow's milk protein allergy (CMPA) has a wide prevalence with the overall occurrence in the population at 2–7% in different countries (Høst, 2002; Shriver, & Yang, 2011). As indicated above, milk proteins are composed of variety of proteins but only a few are known to be allergenic (Hochwallner, Schulmeister, Swoboda, Spitzauer, & Valenta, 2014). The prevalent cow's milk proteins involved in allergic responses in children include β-lactoglobulin, α-lactalbumin, α_s-casein, β-casein and κ-casein. Children affected by CMPA react to a specific fraction of milk proteins that contains specific epitopes widely spread along the protein molecules. In general, a study of the molecular characteristics

of a known protein allergen allows for identification of processes that may be capable of minimising allergenicity thus allow for development and production of hypoallergenic formulas or improving the tolerance of allergic subjects to these proteins (Restani, *et, al.*, 2004). Avoidance of foods containing milk proteins has been one of the approaches in the management of CMPA, however, this is not a recommended practice due to the importance of these proteins in the diet. Fortunately, the allergic properties of many proteins are diminished by enzymatic hydrolysis with digestive proteolytic enzymes. By their action, a native structure of proteins is cleaved and changed followed by a release of small peptides and free amino acids. Diminished allergenicity is either due to cleaving of an epitope or a change in the conformation (Nasirpour, Scher, & Desobry, 2006; Noman, *et, al.*, 2018). In many instances, released peptides are hydrophobic which affects the functionality of these hydrolysates, but this approach usually incurs a higher cost (Exl, & Fritsché, 2001). Regardless these shortcomings, infant formulas containing fully- or partially-hydrolysed milk proteins are recommended as a first alternative for children with CMPA (El-Agamy, 2007). While numerous studies have been conducted, research is still ongoing since these formulas, either extensively or partially hydrolysed formulas, still do not fully meet these expectations (Fritsché, 1998; Pecquet, *et, al.*, 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.

Despite their relatively uncommon allergic potential, milk proteins have substantial applicability in various food products due to their nutritional and/or physical properties. However, they cannot be used over the wide range of typical processing conditions. For example, whey proteins are very soluble however this property is highly dependent on the system pH which can create problems during downstream processing and especially during thermal processing. Thus, partial hydrolysis may improve the stability of whey proteins by

increasing their solubility and their subsequent heat stability. Furthermore, these partial adjustments may improve other functional characteristics including foaming and emulsification. Milk protein isolates, for example, have poor water solubility which limits their functional properties. Thus, protease hydrolysis of MPIs comprised mainly of caseins may potentially improve their functional properties (Ryan, Nongonierma, O'Regan, & FitzGerald, 2018). Several studies have applied a controlled enzymatic hydrolysis to enhance the functional properties of whey proteins, caseins and MPIs. It has been observed that with a greater degree of hydrolysis, solubility can be increased, and viscosity can be decreased (Abd-El-Salam, El-Shibiny, & Salem, 2009). In addition, extensive hydrolysis of whey proteins has been shown to have a negative effect on functional properties, whereas partial hydrolysis of whey proteins results in improvement in heat stability as well as other functional properties (Foegeding, Davis, Doucet, & McGuffey, 2002). Milk protein concentrates have poor emulsification and foaming properties when compared to other milk proteins such as whey, WPC, WPI, and sodium caseinate. As a result, the use of MPCs is limited to processed meats, coffee creamers, whipped toppings and soups (Singh, 2011). Overall, the knowledge and understanding of the enzymatic action on MPCs and the impact on its functionality is limited.

Protein modifications in the food industry are usually achieved by application of enzymes as they provide several advantages including fast reaction rates, mild processing conditions and high specificity in comparison to chemical methods. As allergenic and functional properties of proteins are related to protein conformation, any alteration of these characteristics can inevitably affect extent of allergenicity or functionality. In order to fully diminish allergenicity, milk proteins should be extensively hydrolysed (Nasirpour, *et, al.*, 2006), whereas limited proteolysis can enhance protein functionality.

Proteases play a crucial role as they represent approximately 70% of the total commercially available enzymes globally. Proteolytic enzymes play a specific physiological role in a variety

of complex biological processes. Due to large scale requirements, low-cost demands, environmentally friendly production, and minimal health and safety issues, several plant-based proteases are widely produced including as papain, bromelain and actinidin. In addition, these cysteine hydrolases have high proteolytic activity, good stability at high temperature and a wide working pH range (3.0 to 9.0). These properties are suitable to treat a wide range of substrates and highlight the potential for further exploration in dairy systems to develop numerous value-added products (Edwin, & Jagannadham, 2000; Ghosh, 2005). Although these enzymes have been used in certain food processes to produce hydrolysed proteins from other raw materials such as meat and fish products, there is very limited information on applications to modify the structure of milk proteins.

1.2 Research aims

The aim of this research was to establish experimental conditions to optimise the performance of a selected plant proteases on partially or completely hydrolysed reconstituted milk protein preparations to achieve enhanced hypoallergenicity or functionality. Therefore, the specific objectives were to:

- Establish a knowledge base in relation to the optimum enzymatic activity of commercially available plant proteases;
- Determine kinetic and thermodynamic parameters of these proteases on selected milk protein ingredients;
- Determine the extent and specificity of proteolysis in relation to expression of allergenicity;
- Ascertain conditions for limited proteolysis of selected milk protein systems with enhanced functionalities.

1.3 Thesis outline

This thesis has been organised into seven chapters. Chapter 1 provides a brief introduction and presents the aims, significance, and contribution to the existing knowledge and practice of this study. Chapter 2 critically reviews the literature relevant to the thesis and provides an overview of plant proteases including their characteristics and specificity towards different substrates. Chapter 3 details the application of actinidin in the hydrolysis of milk proteins to establish operational parameters (temperature, enzyme to substrate ratio and time). Chapter 4 discusses the role of actinidin in the hydrolysis of milk proteins in relation to minimising allergenicity. Chapter 5 encompass the behaviour of actinidin in relation to solubility, heat stability, emulsification and foaming of milk and whey protein concentrates. Chapter 6 determines the kinetics and thermodynamic parameters of actinidin, bromelain and papain in milk protein hydrolysis. Chapter 7 concludes the research findings and reiterates the consequences and significance of the study. This chapter also explains the limitations of the study including final suggestions for future studies.

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Chapter 2

Literature Review Part A

A review of the literature is presented here in two parts. Part A, Sections 2.1-2.7, provides a review of the various components of milk, and the proteases that are commonly used to alter the structural characteristics of dairy proteins. Various mechanisms are also discussed. In Part B, Sections 2.8, a comprehensive review of plant proteases and their application in dairy systems is presented. This section has been published in International Dairy Journal.

2.1 Bovine milk and dairy proteins

2.1.1 Bovine milk

Milk is a very complex liquid and is defined as an aqueous solution of lactose, milk proteins (dispersed colloidal particles), salts (inorganic and organic), and fats (emulsified lipid globules) (Walstra, Jenness, & Badings, 1984). Milk proteins are highly valuable nutritional components required for the proper development of infants and toddlers and maintenance of muscles in the elderly. The average composition of the main components of bovine milk (Table 2.1) includes 87% water, 4.6% lactose, 3.3% proteins and 4% lipids, mineral substances (0.65 %) and organic acids such as citrate, enzymes and vitamins (Walstra, *et al*, 1984; Walstra, Wouters, & Geurts, 2005).

Table 2.1: Composition of bovine milk

adapted from Walstra, *et, al.*, (1984) and Walstra, *et, al.*, (2005)

	Average content (%w/w)		
Component	**Serum Phase**	**Colloidal Phase**	**Total**
Water	**79**	**8**	**87**
Protein	**0.56**	**2.67**	**3.23**
Casein	-	2.6	2.6
α_{s1}-Casein	-	1.02	1.02
α_{s2}-Casein	-	0.28	0.28
β-Casein	-	0.93	0.93
κ-Casein	-	0.37	0.37
Whey protein	0.56	-	0.56
α-Lactalbumin	0.12	-	0.12
β-Lactoglobulin	0.32	-	0.32
Immunoglobulins	0.08	-	0.08
Bovine serum albumin	0.04	-	0.04
Carbohydrates (lactose)	**4.6**	**-**	**4.6**
Lipids	**0.016**	**4.058**	**4.07**
Minerals	**0.481**	**0.2**	**0.7**
Organic acids	**0.161**	**0.026**	**0.189**
Other		**0.14**	

2.1.2 Milk proteins

The terms “protein” and “casein” were introduced by Mulder in 1838 and Broconnet in 1830 respectively, and in 1814 the first research paper on milk proteins was published by Berzelius (Huppertz, Fox, & Kelly, 2018). Milk proteins are well known for their nutritional, biological and functional properties in the food and pharmaceutical industries. They were originally consumed by athletes but are now known to be beneficial for the general population and any age group. Milk proteins are comprised of caseins and whey proteins and since caseins are nutritionally valuable, they are the main protein used in the production of various food products. Whey proteins are abundant in branched chain amino acids (BCAA), which play a crucial role in the human diet by providing these important amino acids for muscle physiology. Whey proteins are a co-product (traditionally known as by-product) in the production of the majority of cheeses and caseinates. Whey proteins present a very diverse group of proteins including α-lactalbumin, β-lactoglobulin, proteose peptone, lactoferrin, lactoperoxidase, immunoglobulins and serum albumin. In addition to muscular health, whey proteins and their peptides have many other health benefits that can improve immune, cardiovascular, digestive and nervous systems. They can be used in many applications such as drying aids, pharmaceutical (directly and as excipients), films, coatings and specialised nutrition (Ghosh, Prasad, & Saha, 2017). Caseins exist in milk as large colloidal complexes with calcium phosphate known as micelles with diameters 150-200 nm. These micelles are heat-stable and can be heated at 100 ºC for 24 hours or at 140 ºC for up to 25 minutes without coagulating, whereas whey proteins are very heat sensitive (Fox and McSweeney, 2003).

2.1.3 Caseins

Caseins represent the largest fraction of bovine milk (about 80%) and are comprised of α_{s1}-, α_{s2}-, β- and κ- forms with an approximate availability in milk of 10, 2.6, 9.3 and 3.3 g/L,

respectively. The main properties of all four fractions are listed in Table 2.2 and although caseins have well defined primary structures, their secondary and tertiary structures have not been fully understood.

Table 2.2: Main characteristics of casein fractions

adapted from Huppertz (2013) and Fox, Uniacke-Lowe, McSweeney, & O'Mahony (2015)

Characteristic	**α_{s1}-Casein α_{s1}-CN B-8P**	**α_{s2}-Casein α_{s2}-CN A-11P**	**β-Casein β-CN A2-5P**	**κ-Casein κ-CN A-1P**
Molecular weight (Da)	23599	25206	23973	19052
Number of amino acid residues	199	207	209	169
Serine	16	17	16	13
Proline	17	10	35	20
Cysteine	0	2	0	2
Lysine	14	24	11	9
Positively charged residues	25	33	20	17
Negatively charged residues	40	39	28	28
Aromatic residues	20	20	14	14

2.1.3.1 α_{s1}-Casein

The α_{s1} form of casein (α_{s1}-CN) is the major protein fraction representing approximately 40% of the total caseins in bovine milk (Huppertz, 2013). It has a molecular weight of ~23.6 kDa and 199 amino acid units where its sequence contains Asp (7), Asn (8), Thr (5), Ser (8), SerP (8), Glu (24), Gln (15), Pro (17), Gly (9), Ala (9), Val (11), Met (5), His (11), Leu (17), Tyr (10), Phe (8), Trp (2), Lys (14), His (5) and Arg (6) (Swaisgood, 1982). It has two predominantly hydrophobic regions and one highly charged polar zone (Fox, Uniacke-Lowe, McSweeney, & O'Mahony, 2015). It lacks a well-defined secondary structure due to the presence of a relatively high proline content and it has ~8 genetic variants with 8-9

phosphorylated amino acid residues where the majority of them are serine. It lacks cysteine residues and is a calcium sensitive fraction precipitating at 3-8 mM $CaCl_2$. At pH 6.6 and ionic strength of 0.003, α_{s1}-CN exists as a monomer. As the ionic strength increases to 0.01, a monomer-dimer equilibrium is formed and enhancing the ionic strength further to 0.2 facilitates the formation of dimers and tetramers. However, the degree of association increases once the pH level rises above 6.6. From and allergenicity perspective, in the case of α_{s1}-CN, the specific protein fragments including f(21-35), f(56-70), and f(161-175) are considered reactive epitopes as these were recognised by IgG antibodies (Cong, *et, al.*, 2013). The primary sequence of f(21-35) is Leu-Arg-Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys-Glu; f(56-70) consists of Asp-Ile-Lys-Gln-Met-Glu-Ala-Glu-Ser-Ile-Ser-Ser-Ser-Glu-Glu; and f(161-175) consists of Ser-Gly-Ala-Trp-Tyr-Tyr-Val-Pro-Leu-Gly-Thr-Gln-Tyr-Thr-Asp (Cong, *et, al.*, 2013).

2.1.3.2 α_{s2}-Casein

This protein fraction is characterised as the most hydrophilic due to the presence of highest number of phosphorylated residues (10-13) among all caseins and represent approximately 10% of the total caseins in bovine milk. This protein has 207 amino acid residues in its polypeptide chain with molecular weight of ~25.2 kDa. The amino acid sequence contains Asp (4), Asn (14), Thr (15), Ser (6), SerP (11), Glu (25), Gln (15), Pro (10), Gly (2), Ala (8), Cys (2), Val (14), Met (4), His (11), Leu (13), Tyr (12), Phe (6), Trp (2), Lys (24), His (3) and Arg (6) (Swaisgood, 1982). The C-terminal is positively charged (a net charge of + 9.5 at pH 6.6) and N-terminal is negatively charged (a net charge of − 21 for the first 68 residues). The higher number of net negative charge clusters makes this fraction more sensitive to changes in ionic strength and cation concentration (i.e., Ca^{2+}) (Kalyankar, Khedkar, Patil, & Deosarkar, 2016).

2.1.3.3 β-Casein

Beta-casein (β-CN) represents ~35% of the total caseins in milk (Huppertz, 2013), with 209 amino acid residues in its polypeptide chain and a molecular weight of ~24 kDa. The amino acid sequence contains Asp (4), Asn (5), Thr (9), Ser (11), SerP (5), Glu (18), Gln (21), Pro (35), Gly (5), Ala (5), Val (19), Met (6), His (10), Leu (22), Tyr (4), Phe (9), Trp (1), Lys (11), His (5) and Arg (4) (Swaisgood, 1982). It is also amphiphilic (the most hydrophobic fraction of caseins), consisting of apolar C-terminal regions and a highly negatively charged N-terminal region. It has a high number of proline residues (Table 2.2) that govern its interactions and it is more temperature dependent but less sensitive to ionic strength (Swaisgood 2003).

2.1.3.4 κ-Casein

Kappa-casein (κ-CN) is the smallest fraction of the caseins and has 169 amino acid residues in its polypeptide chain with a molecular weight of ~19.0 kDa. The amino acid sequence contains Asp (4), Asn (7), Thr (14), Ser (12), SerP (1), Glu (12), Gln (14), Pro (20), Gly (2), Ala (15), Cys (2), Val (11), Met (2), His (13), Leu (8), Tyr (9), Phe (4), Trp (1), Lys (9), His (3), Arg (5) and PyroGlu (1) (Swaisgood, 1982). It is calcium insensitive with low levels of phosphorylation and has the unique feature of being glycosylated (Huppertz, 2013). It has both hydrophilic glycosylated C-terminal regions (with only a few apolar and no aromatic residues) and hydrophobic N-terminal regions that represent its amphipathic nature and presence on the surface of micelles. The overall stability depends on the size of micelle (Swaisgood, 2003), and various enzymes including chymosin can hydrolyse this fraction rapidly at Phe (105)-Met (106) and render N-terminal regions (para κ-CN) with two cysteine residues and C-terminal (macropeptide) regions including all carbohydrate and phosphate groups (Huppertz, 2013).

2.1.3.5 Casein micelles

The main structural feature of casein micelles is their hierarchical organisation of substructures in the form of primary casein particles (PCPs) stabilised by calcium phosphate nanoclusters (Huppertz, Fox, & Kelly, 2018). The micelles have a near-spherical shape and a hydrodynamic radius of 60–120 nm (De Kruif, & Huppertz, 2012) and are sterically stabilised in milk by a polyelectrolyte brush of κ-CN molecules on the surface that have hydrophilic C-terminal and hydrophobic N-terminal regions (Huppertz, *et, al.*, 2018). Basically, these conformations have a 'sponge' like structure with an inhomogeneous water and protein distribution in the core of the micelle (De Kruif, *et, al.*, 2012).

2.1.4 Whey protein fractions

The principal components of whey proteins are α- lactalbumin (ALA; ~ 1.2 g/L), β-lactoglobulin (BLG; ~ 3.2 g/L), bovine serum albumin (BSA; ~ 0.4 g/L), immunoglobulins (Ig; ~ 0.8 g/L), proteose peptones (PP; ~ 0.5 g/L) and minor components comprised of lactoperoxidase, lysosome and lactoferrin (Anfinsen, 1967; Dupont, Croguennec, Brodkorb, & Kouaouci, 2013).

2.1.4.1 β-Lactoglobulin (BLG)

Representing ~50% of the globular whey proteins, BLG is the most abundant of the globular forms and represents about 10% of total proteins in milk. It is comprised of 162 amino acid residues in its polypeptide chain with a molecular weight of ~18.3 kDa. The amino acid sequence contains Asn (5), Ser (7), Gln (9), Gly (4), Pro (8), Ala (15), Val (9), Ile (10), Leu (22), Phe (4), Thr (8), Lys (15), Met (4), His (2), Trp (2), Glu (16), Cys (5), Arg (3), Tyr (4), and Asp (10) (Morr, & Ha, 1993). Numerous studies have been reported on the secondary structures of BLG using a range of different methods. A study conducted by Dong, *et, al.*, (1996) utilised Fourier transform infrared (FTIR) spectroscopy and revealed that the secondary

structure of BLG is composed of α-helix (9-11%), β-sheet (51-55%), β-turn (20-27%) and random coil (9-11%) motifs. Another study performed circular dichroism (CD) spectroscopy and reported its secondary structure comprised of α-helix (15%), β-sheet (50%) and reverse turn structures (15-20%) (Creamer, Parry, & Malcolm, 1983). The secondary crystal structure of BLG has nine strands (A to I) of two anti-parallel β-sheets. Eight strands wrap around to make a flattened β-barrel structure which covers the thiol group with the assistance of α-helix located parallel to the strands of A, G, F and H, and the ninth strand forms the dimer interface. The Cys121 residue of the amino acid sequence of BLG remains as a free thiol (SH) group whereas other four, Cys106-Cys119 and Cys66-Cys160 form two disulfide (SS) bonds (Figure 2.1) (Considine, Patel, Anema, Singh, & Creamer, 2007; Morr, *et, al.*, 1993).

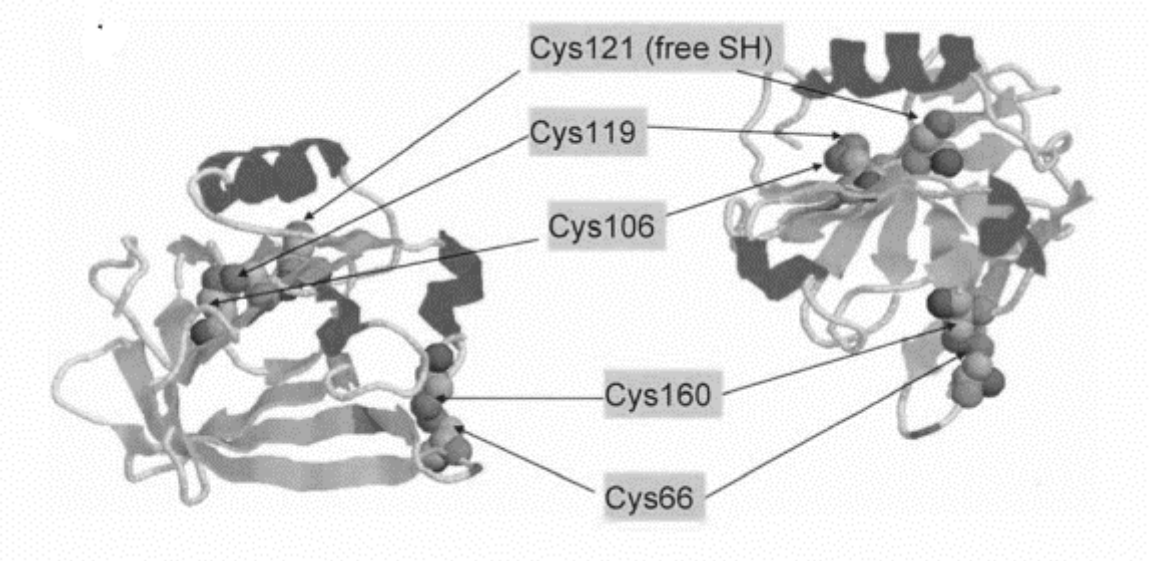

Figure 2.1: Three-dimensional structure of BLG

adapted from Patel, 2007

Of the ten known genetic variants of BLG, BLG A and B are the two main variants that also possess allergenic potential and differences at amino acid positions such as Asp64;Val118 and Gly64;Ala118 for BLG A and B respectively. In the case of β-LG, the main allergenic epitopes considered for this antigen are f(41–60), f(102–124) and f(149–162) (Bogahawaththa, *et, al.*, 2017). The f(41–60) epitope consists of Val-Tyr-Val-Glu-Glu-Leu-Lys-Pro-Thr-Pro-Glu-Gly-

Asp-Leu-Glu-Ile-Leu-Leu-Gln-Lys and is present in the β-strands and located on the surface. The f(102-124) epitope has the following structure: 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). The f(149–162) epitope consists of 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) (Fox, 2003). Along with allergenic potential, these variants also contribute towards difference in their solubility profiles (de Wit, 2009; Fox & McSweeney, 2003; Maier, Okun, Pittner, & Lindner, 2006), and the heat stability also differs with the positioning of salt bridges and amino acids present. For instance, BLG-A has better hydrophobic packing compared to BLG-B that increases its heat stability at higher temperatures (de la Fuente, Singh, & Hemar, 2002). The pH also plays a crucial role in affecting the denaturation temperature of BLG as it becomes most heat stable at pH 6.0 and heat sensitive at pH 4.0 (Sawyer, 2003).

2.1.4.2 α-Lactalbumin (ALA)

Alpha-lactalbumin is the second most abundant whey protein fraction (~20 %) representing about 3.5% of the total protein in milk. It has 162 amino acid residues in its polypeptide chain and four SS bonds with an isoelectric point of at about pH 4.8 and a molecular weight of ~14 kDa. The amino acid sequence contains Trp (4), Asn (12), Tyr (4), Ser (7), Gln (5), Gly (6), Arg (1), Cys (8), Ile (8), Lys (12), Ala (3), Asp (9), Val (6), Thr (7), Met (1), His (3), Leu (13), Glu (8), Cys (8), Phe (4), and Pro (2) (Morr, *et, al.*, 1993) and major epitope regions for this protein fraction are f(7-18), f(53-62) and f(89-108) (Jarvinen, *et, al.,* 2001).

An investigation using CD spectroscopy as well as X-ray crystallography revealed secondary structures of ALA are composed of unordered structures (60%), α-helices (20%) and β-sheets (14%) (Patel, 2007; Robbins, & Holmes, 1970). A-LA has two separate domains, the α-domain

and the β-domain, where the former domain is rich in α-helixes and has two SS bonds (i) between Cys-6 and Cys-120 and (ii) between Cys-28 and Cys-111. The β-domain is rich in β-sheets also has two SS bonds (i) between Cys-61 and Cys-77 and (ii) between Cys-73 and Cys-91 (Brownlow, *et, al.*, 1997; Considine, *et, al.*, 2007). Unlike BLG, ALA lacks free SH groups and its pure fraction without BLG will inhibit gel formation and is thus more heat stable (Corredig, & Dalgleish 1999).

2.1.4.3 Bovine serum albumin (BSA)

Bovine serum albumin is comprised of 582 amino acid residues in its polypeptide chain with an isoelectric point at about pH 5.3 and a molecular weight of ~66 kDa (Damodaran, Parkin & Fennema 2008; Morr, at al., 1993). The amino acid sequence contains Asn (12), Thr (34), Glu (59), Glx (1), Gly (16), Cys (35), Met (4), Leu (61), Phe (27), Lys (59), Trp (2), Asp (39), Gln (19), Asx (3), Ser (28), Val (36), Pro (28), His (17), Arg (23), Tyr (19), Ile (14), and Ala (46) (Morr, *et, al.*, 1993). It has three domains stabilised by 17 intramolecular disulfide bonds resulting in an oblate shape, and a free thiol group at the Cys34 residue (Considine, *et, al.*, 2007; Thompson, Boland, & Singh, 2009). BSA consists of helices, turns, and extended chains but it lacks β-sheets (Considine, *et, al.*, 2007). The N-terminal region of its molecule is less compact compared to the C-terminal region and the different domains indicate differences in net charge, ligand binding properties and hydrophobicity (Morr, *et, al.*, 1993).

2.1.4.4 Lactoferrin (LF)

Lactoferrin is comprised of 689 amino acid residues and is a monomeric protein with a molecular weight of ~80 kDa. It is an iron binding glycoprotein and has two lobes with each capable of reverse chelating two ferric ions. Both lobes the have the same folds with a consistent sequence (~40%), and each lobe has two domains with the N-lobe (N1 and N2) and the C-lobe (C1, C2) each with an iron binding site present. It has 16 intramolecular disulfide

bonds without any free thiol groups (Lonnerdal, & Suzuki, 2013; Madureira, Pereira, Gomes, Pintado, & Malcata, 2007).

2.1.4.5 Immunoglobulins (Ig)

Immunoglobulins, along with LF, BSA and proteose peptone, are minor protein fractions present at the serum (Fox, *et, al.*, 2015). The Ig types are globular proteins that are also antibodies that can be classified into three categories IgM, IgA and IgG. The IgA and IgM monomers are similar to IgG with only difference the presence of a C-terminal octapeptide on the heavy chain. Together, IgG1 and IgG2 form IgG with IgG1 the major component (~80%). An Ig molecule has a molecular weight of ~160 kDa and the structure contains two identical heavy chains and two identical light chains with molecular weights of ~53 kDa and ~23 kDa respectively that are connected by disulfide bonds. The SS binding location and distance between heavy chains varies with type of Ig. Each molecule forms two identical antigen binding sites by the N-terminal part of one heavy and light chain appearing as a Y-shaped molecule (Gapper, Copestake, Otter, & Indyk, 2007; Hurley, & Theil, 2013; Hurley, & Theil, 2011; Korhonen, Marnila, & Gill, 2000).

2.1.5 Protein interactions

Protein-protein interactions occur due to the presence of either covalent or non-covalent interactions depending on the environmental conditions. Non-covalent interactions are molecular and steric repulsions, hydrophobic interactions, van der Waals forces, hydrogen bonds and depletion interactions, whereas covalent interactions exhibit electron sharing and stronger bonds such as disulphide bonds (Walstra, Wouters & Geurts 2006).

2.2 Production of milk protein hydrolysates

Short chain peptides and free amino acids are hydrolysates that can be obtained by the process of protein hydrolysis (proteolysis). Milk protein hydrolysates can be obtained via various chemical and biochemical methods such as acid, alkaline, microbial enzymatic or digestive enzymatic hydrolysis (Ovissipour, *et, al.*, 2012). The former chemical techniques have disadvantages as their use may render food products unsuitable. Therefore, enzymatic hydrolysis is a better technique for the production of functional and nutritional products as it is faster can be better controlled (Noman, *et, al.*, 2018). Under optimum conditions, milk peptides liberated during hydrolysis are mainly depend on the type of hydrolysis method used, pH, incubation time and temperature (Mendis, Rajapakse, & Kim, 2006; See, Hoo, & Babji, 2011).

2.2.1 Chemical hydrolysis

Chemical hydrolysis is a rapid method for hydrolysing milk into by- or co-products. Milk proteins are hydrolysed into different peptides under either alkaline or acidic conditions (Batista, 1999; Gao, Hirata, Toorisaka, & Hano, 2006). Chemical hydrolysis method is very economical to implement with the use of higher temperatures, strong chemicals and extreme pH resulting in faster processing with high yields (Kristinsson, & Rasco, 2000). However, these harsh conditions may increase the bitterness and decrease protein functionality which limit its use in certain food and nutraceutical applications (Sanmartín, Arboleya, Villamiel, & Moreno, 2009).

Early acid-hydrolysis methods required boiling of protein samples with highly concentrated (6N) sulphuric acid (H_2SO_4) for 18 to 24 hours. However, due to the complexity of removing H_2SO_4 after completion of the process, hydrochloric acid (HCl) under reflux was introduced as an alternative reagent (Adler-Nissen, 1986). However, tryptophan is destroyed by this method which is a major drawback where the recovery of this essential amino acid is required in the

final product (Kristinsson, *et, al.*, 2000; Pickering, & Newton, 1990). Contamination and product degradation are further disadvantages of this method. Scientists continued to develop advanced methods including usage of microwave (to facilitate extensive hydrolysis by increasing temperature), protective agent (to control degradation of valuable amino acids), micro-capillary tubes (for the recovery of tryptophan) and vapour phase hydrolysis system (to control contamination) (Adebiyi, Jin, Ogawa, & Muramoto, 2005; Pickering, *et, al.*, 1990; Tsugita, *et, al.*, 1987).

Amos Herbet introduced alkali hydrolysis using either NaOH or KOH (Jones, 2010). Temperatures ranging from 100 to 180ºC or even higher were introduced for the rapid hydrolysis of proteins into smaller peptides and amino acids. This type of hydrolysis can break ~40% of all protein peptide bonds (Kaye, Weber & Wetzel, 2004). Although a final product with small peptides (~98%) and single amino acids was obtained, several drawbacks remained such as racemisation of L- into D- amino acids that cannot be absorbed in human body and adverse reactions due to α-hydrogen abstraction from amino acids (Kaye, *et, al.*, 2004; Kinsella, & Melachouris, 1976).

A method based on pH adjustment or isoelectric/precipitation was established in late 1990's in United States (at the University of Massachusetts Marine Station) to obtain hydrolysates with improved yield and functional properties (Hultin, & Kelleher, 1999). Chen, Tou and Jaczynski (2007) reported that hydrolysates obtained by this method were of higher quality and suitable for human consumption.

2.2.2 Biochemical hydrolysis

A wide range of proteases exist in nature with different functions and a variety of specificities and protein structures (Krem, Rose, & Di Cera, 2000). Some of these proteases are designed for different applications including those applicable to limited proteolysis which involves

proteases with a very high specificity and the cleavage of a limited number of specific peptide bonds in the protein to yield a slightly modified form of the protein. Other proteases are involved in degradative proteolysis which results in a more extensive hydrolysis of a protein including the hydrolysis of multiple peptide bonds and eventually complete conversion into amino acids (Figure 2.2) (Li, *et, al.*, 2013). Protease specificity is governed by the way it interacts with the substrate to perform its action which is the core of protease applications. Commercial proteases or the use of proteolytic enzymes present in other food products can be used to produce MPH provided that their optimum operating conditions such as temperature, pH, ionic strength, E:S are compatible (Kristinsson, & Rasco, 2000). Diniz and Martin (1997) reported the advantages of enzymatic hydrolysis over other methods includes the possibility to control the characteristics of end product, the use of mild pH and constant temperatures to obtain a better product quality, obtaining end products with improved functional properties, and without the destruction of amino acids.

Proteases (EC 3.4) play a crucial role as they represent approximately 60% of the total commercial enzymes available globally (Gurumallesh, Alagu, Ramakrishnan, & Muthusamy, 2019). Proteolytic enzymes play a specific physiological role in a variety of complex biological processes. For example, in these enzymes, cysteine residues play a nucleophilic role of attacking the peptide bond. Due to large scale requirements and low-cost demands, some plant-based proteases are widely produced such as papain obtained from papaya fruit. However, there is very limited information available for some cysteine proteases including bromelain, actinidin and papain with regard to their capacity to modify the structure of milk proteins. These hydrolysates often have poor flavour and bitter taste profiles (Exl, & Fritsché, 2001), however, they have been used in certain food processes to produce hydrolysed proteins from other raw materials such as meat and fish products. Enzyme specificity is a unique characteristic of the enzymes where they act as a catalyst for only one or a limited number of reactions. Proteases

therefore have a different specificity for various types of peptide bonds that may lead to formation of different amino acid residues at the end and different lengths of amino acid chains.

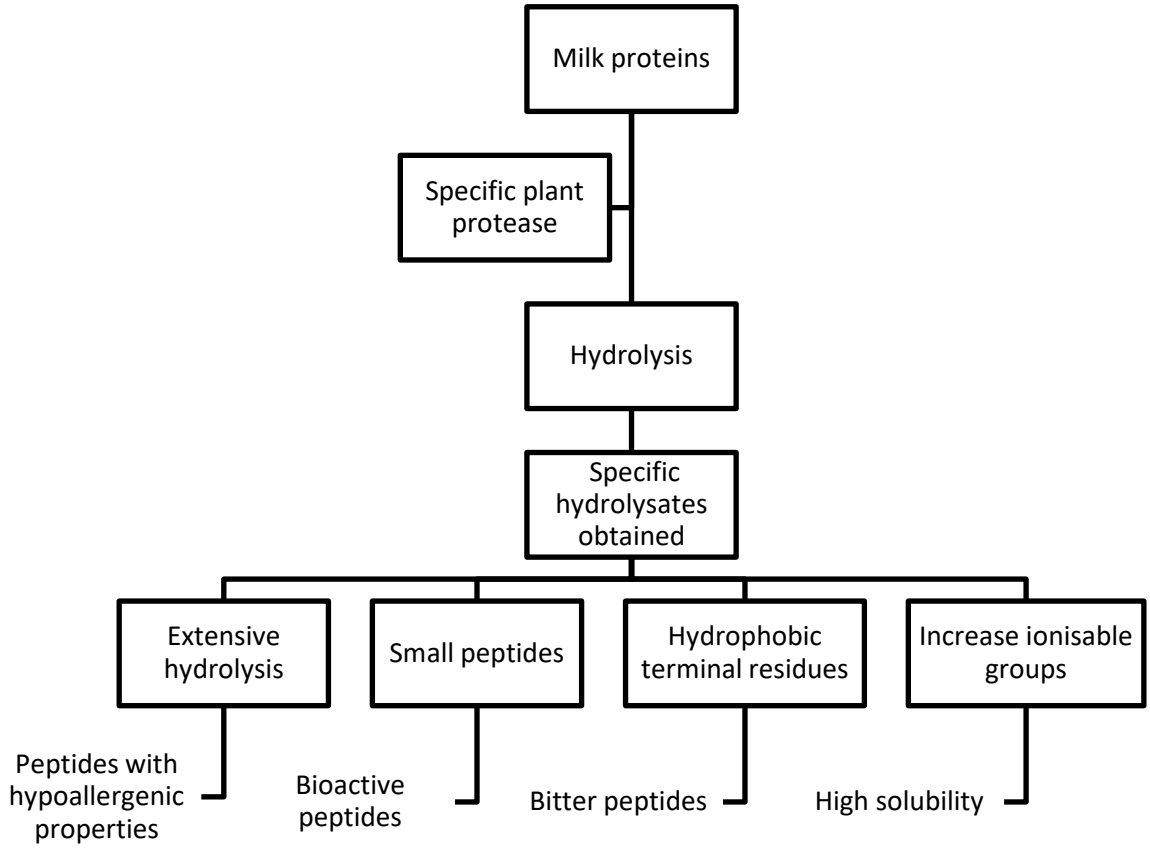

Figure 2.2: Specific properties of proteins/peptides liberated by proteolysis

2.2.3 Classification of peptidases

Three criteria are used in the classification of peptidases:

A. Catalysed reaction: Six main groups are established by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB). Enzymes such as bromelain, actinidin and papain, belong to Group 3 (Hydrolases) and Subgroup 4 (Hydrolases of peptide bonds) (Barrett, 1994).

B. Nature of the catalytic active site: According to Hartley (1960), proteases are divided into six mechanistic classes such as cysteine endopeptidases (EC 3.4.22), also called thiol proteases; serine endopeptidases (EC 3.4.21); aspartic endopeptidases, also known as acid proteases; metallo endopeptidases (EC 3.4.24); threonine endopeptidases (EC 3.4.25) and glutamic endopeptidases (EC 3.4.23). Bromelain (EC 3.4.22.33), actinidin (EC 3.4.22.14), and papain (EC 3.4.22.2) belong to the cysteine endopeptidase class. These three enzymes contain sulfhydryl groups, and their activity depends upon the number of sulfhydryl groups present at their active sites.

C. Structural basis: Proteases are classified according to the amino acid sequence and their relationship in families. The MEROPS database classifies these clans and families by catalytic types denoted by various alphabets such as C, S, T, G, U, M and A, and if a clan belongs to more than one family, they are denoted by the letter P. Cysteine proteases are denoted by CP which includes 0 clans from which bromelain, actinidin and papain belong to Clan CA, Family C1 and Subfamily A (Rawlings, Barrett, & Bateman, 1993).

2.3 Plant based proteases

2.3.1 Production of plant-based proteases

Proteases used in dairy products have been extracted and characterised from almost every part of plants such as seeds, flowers, and latexes. Plant proteases can be extracted either through *in vitro* cultures or directly from their natural source (Nuria, *et, al.*, 2011).

2.3.1.1 Extraction of enzymes from plants

Various plant-based proteases are produced from plant sources such as bromelain, papain, ficin and actinidin. Actinidin is obtained from kiwifruit (EC 3.4.22.14) and is also known as actinidain, *Actinidia* anionic protease and protease B (freesia) (Baker, Boland, Calder, &

Hardman, 1980). Ficin is obtained from fig latex (EC 3.4.22.3) and papain is obtained from papaya latex (EC 3.4.22.2) (Rawlings, Barrett, Woessner, & Salvesen, 2012). Bromelain, also known as *A. comosus*, belongs to the family of *Bromeliaceae* and is obtained from pineapple stems and fruit with the enzymes known as "stem bromelain" (EC 3.4.22.32) and ananase "fruit bromelain" (EC 3.4.22.33) respectively. Hieronymain is another enzyme extracted from *Bromelia hieronymi* fruits (Bruno, Pardo, Caffini, & Lopez, 2003). Proteases have also been extracted from various plant sources such as the seeds of *Solanum dubium*, peeled ginger rhizomes, whole albizia (*Albizia lebbeck*) seeds, and peeled sunflower seeds (*Helianthus annuus*) (Shah, Mir, & Paray, 2014). Column chromatography, ammonium sulfate precipitation and gel filtration are some of the primary methods used to purify extracts of plant proteases, with focusing, affinity chromatography and hydrophobic interaction chromatography some of the secondary techniques.

2.3.1.2 Micropropagation and embryogenesis

Several factors limit the production of proteases such as the high costs of enzyme purification, increasing social demands, land clearing solely for enzyme production, and heterogenicity of extraction sources. To address these limitations, proteases are produced by *in vitro* techniques such as micropropagation and embryogenesis. Micropropagation involves the isolation and treatment of selected axillary buds or shoot tips to facilitate the rapid growth of the plant. This technique has been used to obtain ficin protease from figs (*Ficus carica* L.) (Pasqual & Ferreira, 2007); papain from papaya (*Carica papaya* L.) (Panjaitan, Aziz, Rashid, & Saleh, 2007); and bromelains from *Ananas comosus* Merr. (Teng, 1997).

In somatic embryogenesis, selected shoots are grown on either a callus derived from stem, root and leaf parts or directly on these parts (González-Rábade, Badillo-Corona, Aranda-Barradas, & Oliver-Salvador Mdel, 2011). Papaya hypocotyl callus and seedlings of papaya have been

used for embryogenesis to obtain papain at large scale to meet a growing demand (Fitch, 1993; Usman, et. al., 2002a, 2002b, 2002c).

2.3.1.3 Proteases by cell suspensions and callus cultures

Techniques based on cell suspensions and callus cultures are used to facilitate enhanced enzymatic *in vitro* production where direct tissue extraction by chemical synthesis is limited. Bioreactors, differentiation, elicitation, immobilisation, medium optimisation and metabolic engineering are among the variables reviewed by Roberts and Shuler (1997) used to enhance the productivity of plant cell cultures. Callus cultures, if properly cared for, can be kept for an indefinite period.

Proteases obtained using cell suspension cultures obtained from *Cynara cardunculus, Silybum marianum* and *Centaurea calcitrapa*; and callus obtained from *Mirabilis jalapa, Silybum marianum* and *Cynara cardunculus,* are examples of the more illustrious proteases of plant origins (Shah, at al., 2014). These techniques are advantageous in the event of a scarcity of original plant sources. For an example, *Centaura calcitrapa* yield annual flowers that are used to produce milk clotting enzymes. Furthermore, heterogeneity of the flowers leads to best use of this *in vitro* technique. A study conducted by Tamer and Mavituna (1997) on the production of the milk-clotting enzyme from *Mirabilis jalapa* (ornamental plant with flowers) showed that callus cultures and cell suspension techniques yielded 36- and 54-fold more proteolytic activity than leaves of the original plant respectively in the production of original cheeses in Turkey. Tamer (1993) also reported a 10-fold greater proteolytic activity (milk-clotting) in callus cultures and cell suspension of *Onopordum turcicum* compared to its seeds and leaves parts.

2.3.2 General aspects of plant endopeptidases

Proteases are unique among enzymes and have maintained a robust position in food science since their discovery in the 19th century. Initially, plant proteases were used in the form of

vegetable tissues and crude aqueous extracts. Nowadays, due to advancements in extraction, purification and characterisation techniques, they are used in highly purified forms (Tavano, Berenguer-Murcia, Secundo, & Fernandez-Lafuente, 2018).

Proteases in general are classified as either exopeptidases (EC 3.4.11 - EC 3.4.19) (acting on the ends of the peptide chain) or endopeptidases (EC 3.4.21 - EC 3.4.24; EC 3.4.99) (acting within the molecule) (Palma, *et, al.*, 2002). Plant proteases belong to the class of endopeptidases and are classified by the specificity of their active sites. According to the MEROPS database, proteases are generally categorised into seven classes including asparagine, aspartic, cysteine, glutamic, metallo, serine and threonine, whereas plant-based proteases are categorised into five including cysteine, serine, metallo, aspartic and threonine (Rawlings, *et, al.*, 2009). Additionally, plant-based proteases are classified according to their catalytic mechanism during hydrolysis (Bah, Paulsen, Diallo, & Johansen, 2006), with cysteine and serine protease having a nucleophile present at their active binding site whereas metallo, aspartic and threonine proteases use water molecules (Bruno, Trejo, Avilés, Caffini, & López, 2006).

2.3.2.1 Cysteine proteases (EC.3.4.22)

Cysteine or thiol proteases use Cys residues in their catalytic mechanism and are present in eukaryotes and prokaryotes. This group of plant enzymes consists of papain, actinidin, bromelain, ficin, chymopapain, caricain and aleurain, from which papain is the most widely studied protease (Turk, Turk, & Turk, 1997). There are various other types of cysteine proteases such as papain-like, cathepsin-like, caspase-like and vacuolar-processing (Palma, et. al., 2002). These proteases can withstand a wide range of pH and temperature with various specificities which imparts this class of enzymes with a great potential for applications in food, pharmaceutical and biotechnology industries.

Plant-based cysteine proteases are categorised into five clans (CA, CD, CE, CF and CO) with the majority of belonging to clan CA (the papain family including actinidin and bromelain) (Feijoo-Siota, & Villa, 2011)**.** Proteases from the CA clan must have a targeting sequence (to direct them to specific cellular compartment) and a protein cleavage 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 C1 family (Baker, *et, al.,* 1980; Carne, & Moore, 1978). Their structure consists of β-barrel-like and α-helix separated by a groove (the active site) with Cysteine 25 and Histidine 159 residues on each side of the groove (Figure 2.3). Asparagine 175 (orients His-159 ring) and Glutamine 19 (leads to Cys-25) are two more residues that are also crucial for members of the CA family.

To date, numerous cysteine plant proteases have been isolated and characterised but with limited application in dairy systems. Examples include: actinidin from *Actinidia chinesis* (Kamphuis, Drenth, & Baker, 1985), araujain from *Araujia hortorum* (Obregon, *et, al.*, 2001; Priolo, *et, al.*, 2000), philibertain from *Philibertia gilliessi* (Sequeiros, *et, al.*, 2005), morrenain from *Morrenia brachystephana* (Vairo-Cavalli, Arribere, Cortadi, Caffini, & Priolo, 2003), calotropin from *Calotropis gigantea* (Pal, & Sinha, 1980) funastrain from *Funastrum clausum* (Morcelle, Trejo, Canals, Aviles, & Priolo, 2004), procerain from *Calotropis procera* (Kumar-Dubey, & Jagannadham, 2003), asclepain from *Asclepias speciosa* (Winnick, Davis, & Greenberg, 1940), *A. curassavica* (Liggieri, *et, al.*, 2004), *A. glaucescens* (Tablero, *et, al.*, 1991), *A. fruticose* (Trejo, Lopez, Cimino, Caffini, & Natalucci, (2001), and *A. syriaca* (Lynn, Brockbank, & Clevette-Radford, 1980).

Papain (EC 3.4.22.2), a well-known plant protease from the same family, was the first to be crystallised and is mostly used as a model in the structural determination of other plant proteases. In addition to its protease activity and broad specificity, it also exhibits transesterase, esterase, thioesterase, transamidase and amidase activities (Barbas, & Wong, 1987; Johnston,

1956). It has 212 amino acids, a molecular weight of 23.4 kDa, and three disulfide bridges with one sulfhydryl group. Papain cleaves the peptide bonds of amino acids Ile, Ala, Trp, Val, Phe, Leu and Tyr within the hydrophobic regions (Lorenzo, *et, al.*, 2018). Papain exhibits a preference for amino acids with large hydrophobic side chains at the P2 position, with the exception of valine at the P1' position.

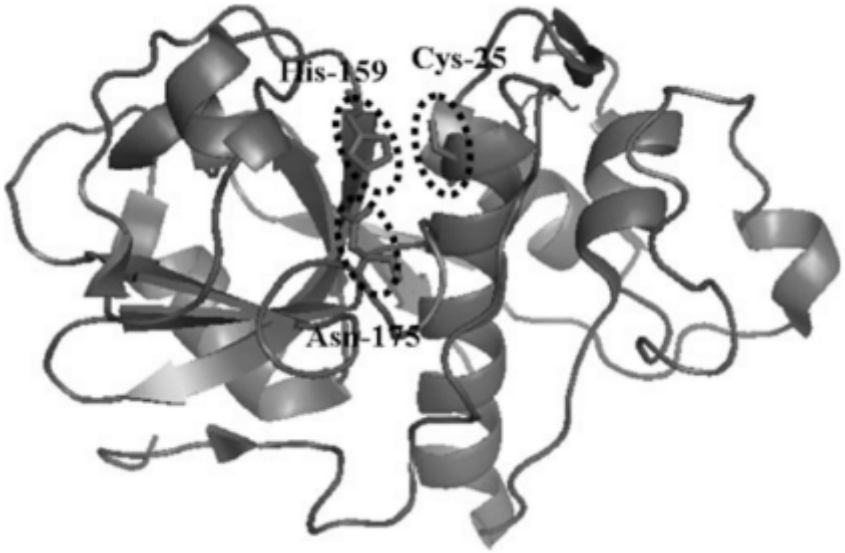

Figure 2.3: Three-dimensional model of papain, PDB code: IPPN

Ottmann, *et, al.*, (2009)

Over recent decades, increasing climate dependency, high costs of papaya crops, and political issues of some of its producing countries have necessitated the search for alternative sources to replace papain (IDEA, 2000. Commercialisation Bulletin 13 Papain Report). Actinidin and bromelain belong to the same family as papain, and all have a titratable free sulfhydryl group, which is essential for their activity. X-ray crystallographic analysis of the three-dimensional structure of actinidin has shown that the polypeptide chain conformation of actinidin is similar to that of papain (Drenth, Jansonius, Koekoek, & Wolthers, 1971). Actinidin can therefore be used as a replacement for papain depending on its specificity with the target substrates.

Actinidin is isolated from kiwifruit (EC 3.4.22.14) and belongs to Clan CA, Family C1 Cysteine and Subfamily A. It is also known as actinidain, *Actinidia* anionic protease and is a thiol protease with 220 amino acids had a molecular weight of 23.5 kDa (Baker, *et, al.*, 1980). An extensive homology has been found in the amino acid sequences, substrate specificity and tertiary structures of actinidin and papain which are from the same family (Baker, *et, al.*, 1980; Carne, *et, al.*, 1978). The actinidin amino acid sequence contains a total of seven cysteines, with one is located inside the active site and the remaining six involved in the formation of three disulphide bridges. The polypeptide chain of actinidin is folded into α-helices and twisted β-sheets, from which the α-helix domain consists of residues from 19 to 115 and 214 to 218, and the β-sheets contains residues from 1 to 18 and 116 to 213. This type of folding arrangement leads to the cleavage occurring between both domains. The amino and carboxylic groups attached at the ends of both domains are crossed over each other leading to the formation of "belts" and further stabilisation of actinidin. Histidine 162 and Cysteine 25 are two residues present at the active site behind the cleft in the middle of the domains (Grozdanović, Gavrović-Jankulović, & Drakulić, 2013; Rawlings, *et, al.*, 2012). The active site 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 reacting substrate (P1, P2, P3, P4, P1', P2' and P3'). The 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}, and the interaction of subsite S2 towards P2 of the substrate provides a major contribution towards actinidin specificity. In actinidin, Met_{211} is present at the lower part of the binding pocket of the S2 subsite, but its side chain changes position during the creation of an actinidin substrate complex, which allows sidechains of Phe residues to approach the subsite. During the hydrolysis process, actinidin mostly cleaves amino acids present on hydrophobic sites of the P2 residue such as Val, Phe or Leu (Boland, & Singh, 2013). Studies have shown that actinidin-induced pre-hydrolysis of proteins resulted in enhanced gastric (Montoya, *et, al.*,

2014) and intestinal (Kaur, Rutherfurd, Moughan, Drummond, & Boland, 2010) digestion. Actinidin is used as a meat tenderiser due to its proteolytic action on myofibril or collagen proteins (Christensen, *et, al.*, 2009; Zhu, Kaur, Staincliffe, & Boland, 2018) and its use results in a better structure of meat compared to papain (Nishiyama, 2007). Actinidin can also be used as a beer clarifier and milk coagulating agent (Zhang, Sun, Liu, Li, & Jiang, 2017), however, there is still only limited information available for the application of actinidin in dairy systems.

Bromelain is obtained from stem (EC 3.4.22.32) and fruit (EC 3.4.22.32) of pineapple, and it resembles papain and actinidin in terms of substrate specificity (Rawlings, *et, al.*, 2012). Fruit bromelain exhibits a broader specificity and higher proteolytic activity compared to stem bromelain (Polaina, & Maccabe, 2007). It exhibits an optimum pH of 6.0-8.5 and temperature of 50-60 °C, and similarly, ficin (EC 3.4.22.3) exhibits an optimum pH of 5.0-8.0 and temperature of 45-55 °C (Polaina, *et, al.*, 2007). Thus far, only N-terminus, Histidine and Cysteine sequencing have been studied in case of ficin with Cys showing homology with papain sequencing. A study conducted by Devaraj, Gowda, & Prakash (2008) revealed enzymatic specificity of ficin towards hydrolysing C-terminal peptide bonds to Glu, Leu and Phe at P_1 position. Ficins obtained from the latex of *Ficus racemose* were shown to degrade caseins and possess milk clotting properties. In this study, ficin exhibited an optimum pH range of 4.5-6.5 at 60 °C. The unique characteristics of these proteases are differentiated according to their composition, chromatography and quantification of essential amino acids at their active sites. For example, ficins obtained from latex of *Ficus anthelmintica, Ficus carica and Ficus glabrata* exhibit many charged forms (Devraj, *et, al.*, 2008) and can therefore be used to obtain different products.

Other cysteine proteases such as chymomexician and mexician are obtained from the latex of the *Jacaratia Mexicana* fruit, and due to their high pH and temperature stability, show high proteolytic activity towards casein substrates (González-Rábade, *et, al.*, 2011). However, in

another study, actinidin was used to hydrolyse MPC and whey proteins and it showed greater substrate specificity towards whey proteins (lactalbumin) than caseins (Kaur, Huppertz, & Vasiljevic, 2021). Of the limited studies using dairy products, one study conducted by Oliveira, *et, al.*, (2019) showed no allergenic reactions in mice allergic to cow's milk after hydrolysing these proteins using *Carica papaya*. Following a two-step hydrolysis, bromelain was used with TGase polymerisation of hydrolysates resulting in a reduction of β-LG capacity to bind with IgE (Villas-Boas, Benede, de Lima Zollner, Netto, & Molina, 2015). In another study, sunflower and albizia seeds were used to obtain extracts to establish milk clotting properties towards bovine caseins (Egito, *et, al.*, 2007).

2.3.2.2 Serine proteases (EC 3.4.21)

Serine proteases (SPs) are one of the largest groups of proteases and Ser is present at the active site of these proteases for binding with the substrates. These proteases have six clans (Rawling and Barrett, 1994) and the majority exhibit extensive homology in reaction mechanism where they share a "catalytic triad" made up of a nucleophile (Serine), an electrophile (Aspartic acid) and a base (Histidine). In the reaction mechanism, the acyl portion of the substrate is transferred to the active side of the protease. First, an ester is formed with the acyl part of the substrate and the Ser oxygen atom leading to the formation of a tetrahedral complex which releases the amino fragment of the substrate. Next, the acyl-protease complex breaks down in the presence of water that releases the final acidic product (Dunn, 2001). This group of plant enzymes consists of kexin types, ClpP and subtilisins proteases, with the majority of plant-based subtilases purified to date members of the pyrolysins (a subfamily of the subtilisins proteases) (Groover, & Jones, 1999) (Figure 2.4). The molecular weight range of serine plant proteases is 60-80 kDa, and they function over wide ranges of pH of (3.0-6.5) and temperature (20-50 °C) (Dunn, 2001).

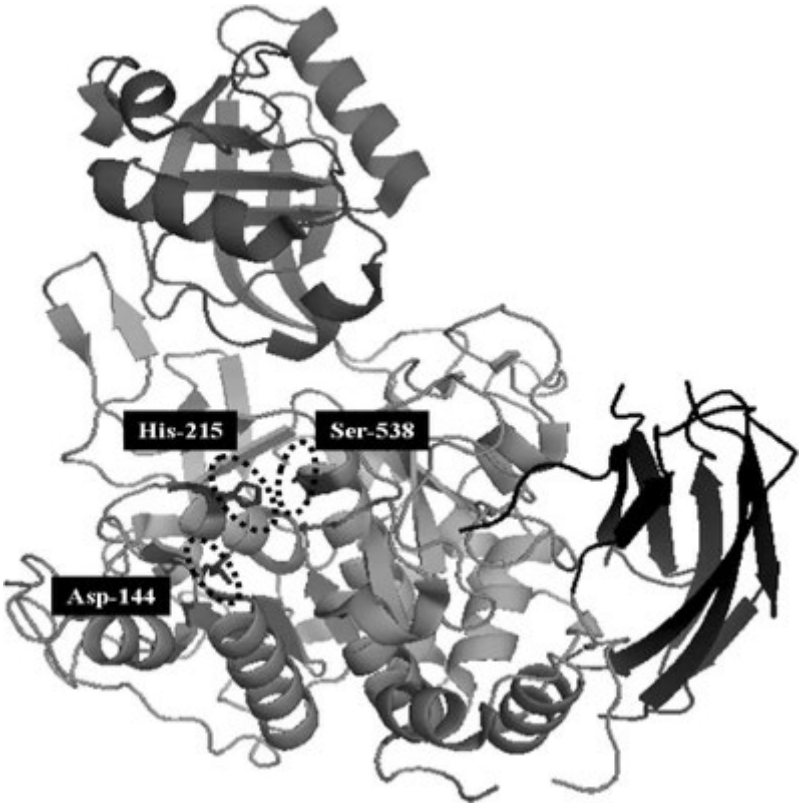

Figure 2.4: Three-dimensional model of serine protease (subtilisin)

PDB code: 3I6S (Ottmann, *et, al.*, 2009)

Cucumisin (EC 3.4.21.25) is obtained from *C. melo* L. varr. Prince and is the first known plant-based serine protease. Peptide sequencing is the preferential approach to study plant proteases from this group and the amino acid sequence of cucumsin is Gly-Thr-Ser-Met. With its active site near the Ser residue, it is similar to subtilisin and is therefore used as a model to determine the ammino acid sequence of other plant serine proteases (Antão, & Malcata, 2005). Cucumsin-like proteases (proteases from the same family exhibiting similar structural characteristics) obtained from the latex of *E. supina* have shown hydrolytic activity towards casein, and seeds from *Cucurbita ficifolia* (a tropical squash) have also been used for casein hydrolysis (Dryjanski, Otlewski, Polanowski, & Wilusz, 1990). In addition, the protease from *C. ficifolia* seeds used in the hydrolysis of dairy substrates have shown a significant reduction in allergenicity (lowest antibody binding capacity) after 24 hours hydrolysis of whey proteins

(WPC-80) and 1 hour for *αs*-casein (Babij, *et, al.*, 2015). Several other serine proteases include protease D from *Cucumsin. melo* L. varr. Inodorus Naud; protease from *C. melo* L. ssp. *melo* varr. Reticulatus; RSIP from *Z. mays* L.; Artocarpin from *A. heterophyllus*; protease A and B from *T. kirilowii*; and serine proteases from *T. bracteate* and *C. cochinchinensis* have been successfully characterized using casein substrates under various conditions (Antão, *et, al.*, 2005).

2.3.2.3 Aspartic proteases (EC 3.4.23)

Aspartic proteases, which are active over an acidic pH range, use an active water molecule at the binding site and are inhibited by pepstatin. Due to the presence of two aspartic residues, the catalytic activity of these enzymes depends upon the specificity for cleavage of hydrophobic amino acid units (Domingos, *et, al.*, 2000). They are obtained from numerous sources including the leaves of tomato and potato plants, potato tubers; seeds of cucumber, squash, rice, barley, hempseed and Arabidopsis thaliana; thistle flowers; and maize pollens (González-Rábade, *et, al.*, 2011). Most aspartic proteases exhibit a greater homology of active sites and primary structures that contribute to their role as coagulants in dairy products at optimum pH. A frequently used example of this protease in dairy is rennet (chymosin) which is used during cheese manufacturing. However, since rennet is obtained from animal sources (calves' stomachs), the high cost and scarcity of rennet has led to demands for its replacement with plant-based substitutes with similar characteristics with thistle flowers one such alternative (Alavi, & Momen, 2020). Cardosin A and B are two active proteases extracted from the flowers of *C. cardunculus* (Figure 2.5) and are present at a ratio of 3:1 in crude extracts. Cardosin A has a molecular weight of 64 kDa and it is comprised of linkages of both light and heavy chains with hydrogen bonds and hydrophobic interactions (Pereira, 2012). Similarly, a mature form of Cardosin B comprised of two chains converted from the proteolysis of precursor single chains has shown around 73% similarity in amino acid sequence to that of Cardosin A and a

higher proteolytic activity (Barros, & Malcata, 2002). Careful consideration should therefore be made before selecting these proteases in cheese manufacturing as the ratio of Cardosin A to B can vary. For example, *Cynara humilis* contains 100% Cardosin A and no Cardonsin B. The aspartic proteases work over a pH range of 2.0-7.0 and a temperature range of 37-65 °C (Veríssimo, *et, al.,* 1996). In dairy systems, these proteases show a preference for polar amino acids in peptide bonds such as Phe-Leu-Ile-Tyr or Phe-Leu-Ile-Val. For example, *C. cardunculus* cleaves the peptide bond at Phe105–Met106, whereas ovine and bovine κ-caseins and cleaves Lys116–Thr117 when caprine κ-casein is the substrate (Sousa, & Malcata, 1998).

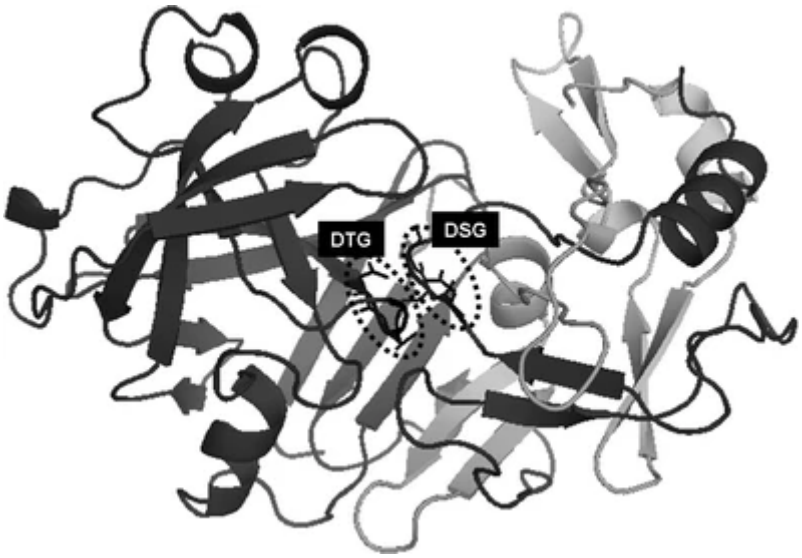

Figure 2.5: Three-dimensional model of aspartic protease (cardosin A)

PDB code: 1B5F. Heavy chain (35kDa) and light chain (15kDa) are denoted by black and grey respectively (Frazão, *et, al.*, 1999).

Other plant proteases that can replace rennet include those derived from artichoke (*Cynara scolymus L.*) (Llorente, Brutti, Natalucci & Caffini, 1997) and *Onopordum turcicum* (Tamer, 1993). Aspartic plant proteases such as cardosins and cyprosins are obtained and purified from the pistils and dried flowers of *Cynara cardunculus,* respectively. Silva and Malcata (2005) established the proteolytic and coagulation activity of cardosins while Heimgartner, *et, al.* (1990) isolated, purified and characterised three types of cyprosins as milk coagulants. A study

conducted by Pino, Prados, Galan, McSweeney and Fernandez-Salguero (2009) reported that plant proteases derived from *C. cardunculus* (cardoon powder) showed greater milk coagulation activity when compared to rennet-derived enzymes.

2.3.2.4 Metalloproteases (EC 3.4.24):

Metalloenzymes possess metal ions (mostly Zn^{2+} or sometimes Co or Mn) for their catalytic activity to hydrolyse proteins. However, MPs are infrequently researched in comparison with other proteases and there is little use for plant derived metalloenzymes in dairy systems to the best of our knowledge (Rawlings, *et, al.*, 2009).

2.4 Degree of hydrolysis

The degree of hydrolysis (DH) is defined as a number of liberated peptide bonds in hydrolysates. It is widely used technique on various proteins and it measures the extent of hydrolysis rates (Adler-Nissen, 1979). Degree of hydrolysis of milk proteins can be determined by various methods as summarised in Table 2.2.

The activity of any enzyme is influenced by several important factors including the substrate concentration, pH, ionic strength, the nature of the ionic environment, and temperature (Palmer, 2001). All of these factors play a role in maintaining or disturbing the conformation of enzymes and/or proteinaceous substrates and thus may either stimulate or inhibit enzyme activity. Variations in environmental pH or ionic quality may alter electrostatic interactions among charged amino acid segments and induce conformational changes in the structure of the enzyme (Palmer, 2001).

Table 2.3: Main methods used in determination of degree of hydrolysis (DH) of milk proteins

Method	Principles	Advantages/disadvantages	References
SN-TCA	Determination of the ratio of 10% TCA soluble nitrogen in the hydrolysates instead of determining the total amount of protein present in sample.	Advantage: A simple method that is useful when working within the pH of 3 to 7 range.	Kumar, Chatli, Singh, Mehta, & Kumar (2016); Homaei, & Samari, (2017); Morais, *et, al.*, (2013); Dąbrowska, *et, al.* (2020)
Formal titration	DH determined from the ratio of α-amino nitrogen and total nitrogen.	Advantage: The increasing number of free amino acids is associated with increasing DH of the hydrolysate (depending on the enzyme used).	Morais, *et, al.*, (2013); Chalabi, Khademi Yarani & Mostafaie, (2014)
Colourimetric (using ninhydrin)	Based on the production of intense colour which serve as the basis of various qualitative and quantitative analytical procedures.	Disadvantage: Less sensitive as compared to OPA method.	Navarrete del T., & García-Carreño, (2003)
TNBS	Based on the reaction of TNBS with primary amines or N-terminal group at slight alkaline conditions.	Advantage: Inexpensive and simple spectrophotometric method.	Dupont, *et, al.*, (2013); Le Maux, Nongonierma, Barre, & FitzGerald, (2016); Adler-Nissen, (1979); Vorob'ev & Kochetkov, (2016); Dąbrowska, *et, al.*, (2020)
OPA	Based on the reaction between amino acid groups with OPA in the presence of beta-mercaptoethanol forming a coloured compound measured at 340nm with spectrophotometer.	Problems: OPA reagent does not react or reacts poorly with proline and cysteine.	Dupont, *et, al.*, (2013); Salami, *et, al.*, (2008); Morais, et al,. (2013); Nazir, Muhammad, Muhammad Khan & Mahrun, (2015)
pH-stat	During hydrolysis, DH is monitored by adding a base to maintain constant pH and the amount of base consumed during hydrolysis is related to no. of peptide bonds hydrolysed.	Problems: Limitations of using this method for pH conditions outside the optimum range. Excessive amount of base present may lead to undesirable end products.	Wróblewska, & Troszyñska, (2005)

2.5 Allergenicity of milk proteins

Food allergies have been recognised as the sixth of the contemporary health problems and milk is considered one of the so called "Big-8" food allergens (Li, *et, al.*, 2011). One of the first encountered food allergies in humans is associated with cow's milk when infants and toddlers are usually first exposed to this food early (Høst, 2002). The cow's milk protein allergy (CMPA) has a wide prevalence with the overall occurrence in the population at 2-7% in different countries (Høst, 2002; Shriver, & Yang, 2011).

The prevention of CMPA requires some important considerations due to the following reasons: (1) CMPA is among the more frequently occurring and most prevalent diseases among infants; (2) cow's milk is good source of calcium for all ages, especially during the growth and development of children; (3) cow's milk proteins are used in various food products as minor or major ingredients which makes it difficult to completely remove from the diet; and (4) the treatment of CMPA incurs a high cost to the health sector. Hence, enhancing knowledge in the field of preventing CMPA by various emerging techniques such as proteolysis can offer a better and more cost-effective alternative to its treatment.

The process of enzymatic hydrolysis by digestive enzymes assists in minimising protein allergies by converting them into short chain peptides and free amino acids. This process alters the proteins so their structure (reactive epitopes) is no longer recognised by antibodies that would initiate an allergenic reaction. However, many properties of these hydrolysates limit their usage in milk products as they can impart a bitter taste, off-flavours, increased osmolality, and a low emulsifying ability. Noman *et, al.*, (2018) reported that specific enzymes can be selected to breakdown antigenic epitopes and can potentially remove bitter peptides. In another study, participants with CMPA showed a significant reduction in allergic response to αs_1-CN from buffalo milk where the degree of hydrolysis was increased using gastrointestinal enzymes such as α-chymotrypsin, trypsin and pepsin (Ahmad, Imran, Khan, & Nisa, 2016).

Around 70 years ago, extensively hydrolysed milk formulas (peptides under 5 kDa) were considered as a treatment for CMPA. However, it was recognised that some newborns and infants require alternatives to breast milk and/or cow's milk even before developing any allergy. Further studies were required to overcome this issue and about 35 years ago, partially hydrolysed formulas (with peptides between 8 to 20 kDa) were developed (Exl, *et, al.,* 2001; Maldonado, Gil, Narbona, & Molina, 1998). The aim was to prevent allergy sensitivity while retaining other properties (such as organoleptic) to achieve a desirable tolerance to cow's milk proteins. Numerous studies have been conducted in the following years, however, allergenicity prevention measures using extensively and partially hydrolysed formulas are still unclear. Further studies are required so that industries can produce formulas with negligible or no allergenic potential (Fritsché, 1998; Pecquet, Bovetto, Maynard, & Fritsché, 2000).

Children with CMPA react to a fraction of milk proteins containing specific epitopes widely spread along the protein molecules. These specific epitopes provoke immune responses such as allergenic reactions (binding with IgE) and/or antigenic reactions (binding with IgG) (Bogahawaththa, Chandrapala, & Vasiljevic, 2017). Therefore, amino acid-based formulas or extensively hydrolysed formulas are recommended for consumption instead of cow's milk as they provide a balanced nutritional profile.

Modern hydrolysed infant formulas vary widely due to the protein source, the degree and type of hydrolysis, the profiles of released peptides (all of which are enzyme type dependant), and other pre- and post-processing methods (Exl, *et, al.,* 2001). For example, a significant reduction of immunoreactive epitopes were observed after the hydrolysis of whey and αs-casein with serine protease from *Yarrowia lipolytica* yeast (Dąbrowska, *et, al.*, 2020). In general, extensively hydrolysed formulas (eHFs) require the use of more than one enzyme. For example, peptides of casein and whey proteins with molecular weights smaller than 2.5 kDa and 8 kDa respectively, were obtained with high nutritional value by hydrolysis using a protease mix

containing *Bacillus licheniformis* enzymes. The potential antigenicity of these peptides was reduced by 103 and 104 times for whey and casein fractions respectively (Boza, Jiménez, Martínez, Suárez, & Gil, 1994).

The first commercially available hydrolysed formula (Beba HA, Good start, NAN HA, Nestle) was introduced in 1985 (Exl, *et, al.,* 2001), and at present, numerous types of infant formulas are commercially available. These are mainly categorised by the processing technique (treatment used), the protein source, and the type of enzymes used (degree and type of hydrolysis) (Fritsché, 1998; Pecquet, *et, al.*, 2000). In many instances, the peptides released are hydrophobic which affects the functionality of these hydrolysates and usually incurs a higher cost (Exl, *et, al.,* 2001). Despite these shortcomings, infant formulas containing fully- or partially-hydrolysed milk proteins are recommended as the first alternative for children with CMPA (El-Agamy, 2007). Partially hydrolysed formulas (pHFs) and extensively hydrolysed formulas (eHFs) for infants are commercially available although some significant differences exist between these formulas especially in the amount of β-lactoglobulin since its level in pHF. So, the pHFs certainly appear to be a better alternative than the general milk-based formula (Exl, *et, al.*, 2001). In addition, the production costs and taste characteristics of pHFs are more favourable than most eHFs (Exl, *et, al.*, 2001). Although numerous studies have been conducted, research is still ongoing since neither eHF nor pHF can currently meet the target requirements (Fritsché, 1998; Pecquet, *et, al.*, 2000).

As summarised in Table 2.3, the main aim of research activities in these areas has been to diminish allergenicity of proteins as well as addressing other properties such as bitter taste, off-flavours, increased osmolality and low emulsifying ability.

Table 2.4: Enzymatic hydrolysis of milk proteins associated with alteration in antigenicity or allergenicity

(Abd-El-Salam, El-Shibiny, & Salem, 2009).

Enzyme	Substrate	Hydrolysis conditions	Immunogenicity reduction	References
Protease of *Bacillus licheniformis*	WPC	Bioreactor temp = 50 ºC, pH = 8.5 and 3 kDa of membrane cut-off.	Antigenicity reduction of 99.97% after hydrolysate obtained with peptides containing 4 amino acids.	Guadix, Camacho, & Guadix, (2006)
Alcalase, papain	WPC	15 mAU/gram protein, temp = 50 ºC, pH = 8, time = 120 mins for (single step) and for two steps its 100 min (first enzyme) + 20 min (second enzyme)	Resulted in reduction in antigenicity (two steps) but with still presence of allergenic epitopes.	Wróblewska, *et, al.*, (2004)
Pepsin, trypsin, chymotrypsin	Lyophilised whey protein	Hydrolysis under higher pressure = 100-300 MPa, pH = 4 (pepsin), pH= 8 (chymotrypsin, trypsin), temp = 37 ºC	In case of pepsin and trypsin, reduction in immune reactivity under HHP hydrolysis was observed. Chymotrypsin did not show any immunoreactivity reduction.	Peñas, Snel, Floris, Préstamo, & Gomez, (2006)
Corolase PP, Corolase PS, Trypsin, Neutrase	β-LG (AA, AB, BB), WPC	pH = 6.5-8.0, E:S = 1:250, Temp = 40-50ºC, obtained hydrolysates were fractioned according to molecular weights.	Lowest antigenicity seen in fraction of 1-5 kDa. β-LG(AA) resulted in lowest response to IgE.	Svenning, *et, al.*, (2000)
Simulated gastric juice	UHT milk	E:S = 2.4:1, temp. = 37 ºC	Immunogenicity retained after hydrolysis with negligible effect on specific IgG and IgE binding with CN epitopes. Digestive stability of β-LG decreased in UHT milk.	Sletten, Holden, Egaas, & Faeste, (2008)

Enzyme	Substrate	Hydrolysis conditions	Immunogenicity reduction	References
Cucurbita ficifolia serine protease	WPC 80 and αs-CN	pH=8, E:S = 150 units per gram of protein, temp. = 37 ºC for up to 24 hours	The lowest IgE and IgG binding response seen for αs-CN (1 hr) and whey (after 24 hrs.)	Babij, *et, al.*, (2015)
Pepsin and trypsin	Native and heated WPC (100 ºC/10 min)	Enzyme (0.05-0.5) to substrate (100) ratio with papsin, pH = 2, temp. = 50 ºC followed by inactivation then hydrolysis with trypsin at pH 8, temp = 50 ºC, time = 120 min	Heated WPC showed lower antigenicity as compared to control at all enzyme levels. Highest reduction in antigenicity for pepsin followed by trypsin at E:S = 0.5:100.	Kim, *et, al.*, (2007)
Alcalase	WPC (77.5% protein)	pH = 7.0–11.0, temp. = 30–60 ºC, E:S = 4000-8000 units/gram of protein	Temperature showed highest impact on anti-αLA-IgG-binding inhibition whereas anti-βLG-IgG-binding inhibition was influenced by pH.	Zheng, Shen, Bu, & Luo, (2008)
Pepsin	β-LG	pH = 2.5, 6.8, temp. = 37 ºC, E:S = 1:20, pressure = 400MPa	Result showed an abrogated allergenicity.	Lopez-Exposito, *et, al.*, (2012)
Pepsin	β-LG	pH = 2.5, 6.8, temp. = 37 ºC, E:S = 1:20, with high pressure	HHP = 400 with hydrolysis process reduced further antigenicity.	Chicón, López-Fandiño, Alonso, & Belloque, (2008)
Four combinations of neutrase, trypsin, protease S	Freeze dried demineralized cheese whey	E:S = 1:100, pH 8.0, temp = 50 ºC, time = 180 min. trypsin/neutrase (1/1) and trypsin/papain (1/1).	Resulted in around 40% of antigenicity reduction.	Shin, *et, al.*, (2007)

Enzyme	Substrate	Hydrolysis conditions	Immunogenicity reduction	References
Alcalase and Bromelain	β-LG	pH = 7.5, temp. = 55 (bromelain) to 60 ºC (Alcalase) followed by polymerization with TGase.	Hydrolysis with or without polymerisation showed β-LG with significant reduction of epitopes and IgE binging capacity.	Villas-Boas, *et, al.*, (2015)
Twelve proteolytic enzymes	WPI	Optimum temperature and pH for each enzyme, time = 5 hours, E:S = 1:100.	IgE immunoreactivity of WPI inhibited with papain (47%) and pancreatin (45%).	Dazeh, (2017)
Latex peptidases	Whey proteins and casein	Temp. = 37 ºC, pH = 6.5, time = up to 24 hours, E:S = 1:15 to 1:45.	Hydrolysates obtained by *Carica papaya* did not show any immune reactivity in allergic mice..	Oliveira, *et, al.*, (2019)
Free & immobilised Alcalase	WPI	Temp = 48 to 62 ºC, time = 180 min, pH = 7.0 to 8.7	Significant reduction of residual allergenicity with free enzyme compared to immobilised.	Pessato, *et, al.*, (2016)
Chymotrypsin, papain, Neutrase, Corolase 7089, Alcalase, Pronase	WPC (78% protein)	E:S = 1:25, temp. = 40 ºC for every enzyme, temp. = 50 ºC (alcalase, corolase, neutrase), time = 5 min	Combination of microwave irradiation with papain, Alcalase or Pronase showed significant reduction of immunoreactivity.	Izquierdo, Peñas, Baeza, & Gomez, (2008)
Alcalase	WPI>90% protein	E:S = 50 or 100 units per gram of protein, pH = 8.5, also used uncontrolled pH, temp. = 60 ºC, time = 180 min	Negligible change in epitope hydrolysates of anti-β-LG, anti-α-LA, IgE and IgG.	Carvalho, Pessato, Fernandes, Zollner, & Netto, (2017)
Danaus plexippus gut peptidases (DpGp)	Casein & β-LG	E:S = 1:20-100, pH = 9.0 & 6.5, temp. = 37 ºC, time = up to 24 hours	Hydrolysates with anti-casein antibodies = no reaction, hydrolysates	Oliveira, *et, al.*, (2018)

Enzyme	Substrate	Hydrolysis conditions	Immunogenicity reduction	References
			with anti-whey protein antibodies = slight reaction.	
Serine protease from *Yarrowia lipolytica*	WPC-80; αs-casein	Temp. = 37 °C, E:S = 1:10, time = 1, 5, 24 hours, pH = 8.0	Inhibition of the reaction was ≤20 (αs-CN) and ≤68% (WPC) after 24 hours.	Dąbrowska, *et, al.*, (2020)
Thermolysin	Casein & WPI	Temp. = 37 ºC, pH = 7.0, E:S = 1:1500, time = 5 min. after heat inactivation, thermolysin, lyophilisation and repolymerisation using TGase	Repolymerised caseins and whey showed negligible and slight (<5%) immunoreactivity respectively.	Damodaran, & Li, (2017)
Pepsin, trypsin, chymotrypsin	Buffalo αs1-casein	E:S = 1:1500, temp = 37 ºC, pH = 7.8 (trypsin, chymotrypsin), pH = 2.2 & 5.5 (pepsin).	Antigenicity reduction for trypsin (85%), chymotrypsin (63%) and pepsin (60%) at pH 2.2 and (38%) at pH 5.5 after 150 min.	Ahmad, *et, al.*, (2016)
Alcalase, trypsin	WPI (~90% protein)	Hydrolysis at temp = 40 ºC, pH = 8 (trypsin) and at temp = 50 ºC, pH 8.5 (alcalase). % DH = 2, 8 and 14% followed by repolymerisation with TGase enzyme.	Significant reduction of antigenicity in case of α-LAand β-LG is seen in case of repolymerization of alcalse than trypsin.	Yu, *et, al.*, (2019)

2.6 Functional properties of milk proteins

The functional properties of milk proteins are related to their molecular hierarchical structure (comprised of primary and secondary forms), which govern the ability of the protein to interact with other components in a food matrix. Therefore, efficient utilisation of milk proteins in food systems depends on tailoring their various structural characteristics (Severin & Xia, 2006). Among other techniques, the functional properties of proteins may be improved by limited proteolysis. For example, limited proteolysis can produce acid-soluble caseins or heat stable whey proteins that are free of off-flavours and are suitable for incorporation into beverages and other foods. Moreover, the treatment can prevent insolubilisation of these proteins and avoid their subsequent precipitation (Singh, 2011).

Solubility is a fundamental prerequisite for other functionalities such as emulsification, gelation, and foaming, and it is therefore a very important to control solubility to achieve desirable end products. Processing parameters such as pH, temperature, ionic strength and protein concentration are important parameters that influence protein solubility (Vojdani, 1996).

Processing issues with MPC such as high viscosity or poor solubility (at room temperature and neutral pH) have resulted in limited utilisation of these concentrates in high energy drinks (Havea, 2006; Singh, 2011). Zwijgers (1992) revealed that increasing the temperature from room temperature to 50 °C improves MPC solubility whereas another study conducted by Babella (1989) revealed a decrease in solubility with the addition of calcium. In a related study, Ye and Singh (2011) showed that MPC solubility was unaffected by the addition of sodium or potassium, however it increased by lowering the level of calcium in the system. Banach and Lamsal (2013) also observed an increase in solubility of MPC after proteolytic hydrolysis with papain, with the control showing around 45% solubility at pH 7 which increased to 70 and 78% after papain hydrolysis for 30 and 180 min, respectively. Similarly, Luo, Pan, & Zhong, (2014)

used papain, pancreatin and trypsin to hydrolyse sodium caseinate and reported that papain exhibited highest solubility with increased degree of hydrolysis.

In general, MPCs are considered to possess good emulsifying and stabilising properties. They act as an emulsifier during homogenisation, facilitate fast adsorption at the interface of oil droplets and form aggregates or single molecules. The resulting steric stabilising protein layers provide stability to the final product by protecting droplets against immediate recoalescence (Dickinson, 1997). Emulsion capacity is typically estimated from oil-in-water emulsions (Hill, 1996) and is defined as the maximum amount of oil that can be dispersed in an aqueous solution containing a given amount of the emulsifier, without reversing into a water-in-oil emulsion or its breakdown.

Poor emulsification issues such as the instability of emulsions with time leads to separation of oil and water phase that further leads to number of issues such as flocculation, creaming and oiling-off, phase inversion and coalescence (Figures 2.6 & 2.7). The emulsion destabilisation process involves one or more of the following: (1) flocculation or aggregation of the droplets to larger units without changing the actual size of each droplet which occurs with weakening van der Waals forces. (2) Creaming and oiling-off which occurs due to extrinsic factors such as centrifugal or gravitational forces. When these forces extend beyond the Brownian motion (thermal motion) of the droplets, a concentration gradient is formed. (3) Sedimentation also occurs due to external forces such as centrifugal or gravitational, where density of medium is lower than its dispersed phase. (4) Phase inversion which as the name suggest is an exchange between the medium and dispersion. For example, this can occur over time when an oil-in-water phase changes into water-in-oil phase. (5) Coalescence which occurs when two or more droplets join together resulting in the breaking or thinning of the film between the droplets (Tadros, 2016). (6) Limited solubility leads to Ostwald Ripening and this can also cause foam destability. In Ostwald Ripening, bigger droplets limit solubility whereas smaller droplet enhances solubility. As time progress smaller droplets solubilise and deposits on larger droplets that increases its particle size/diameter.

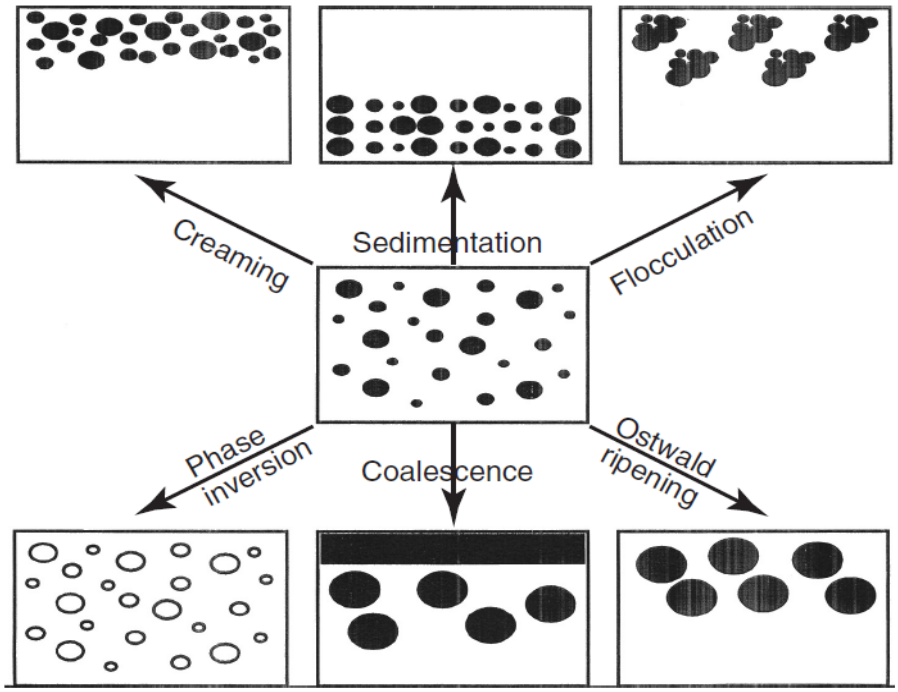

Figure 2.6: Various emulsion breakdown process
adapted from Tadros, (2016)

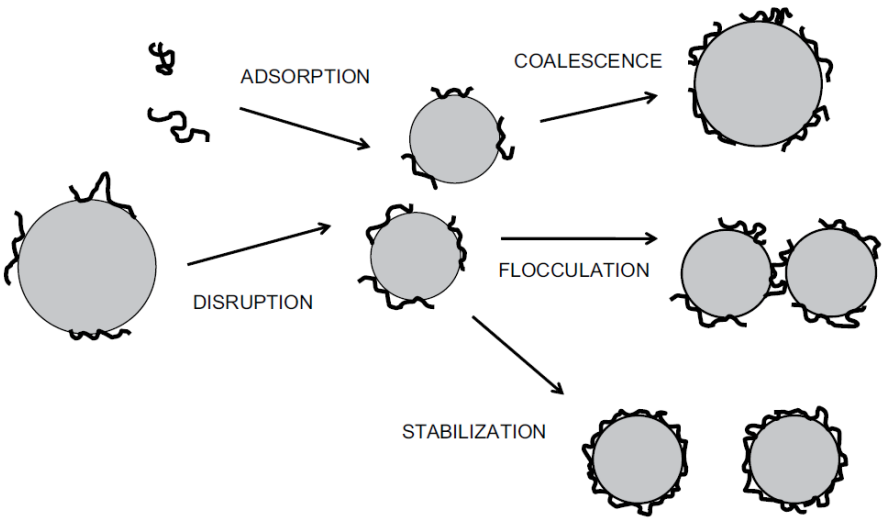

Figure 2.7: Main physico-chemical processes of emulsions formation

Stabilisation of fine droplets requires mechanical disruption of coarse droplets accompanied by rapid effective adsorption of emulsifier at the new oil–water interface. Collision of droplets with limited emulsifier coverage results in coalescence and/or flocculation (adapted from Dickinson, 2008).

Heat stability is another crucial factor determining the overall stability of MPCs which are subjected to reconstitution and heat treatment during processing. Poor heat stability may adversely impact MPC processing which may result in destabilisation of the end product. Various studies have been conducted on MPC focusing on heat stability (Anema, Pinder, Hunter, & Hemar, 2006; Fang, Selomulya, Ainsworth, Palmer, & Chen, 2011; Havea, 2006). For example, κ-CN and β-LG complexes (colloidal or serum) are associated with regions of maximum and minimum heat stability respectively (Oldfield, Singh, Taylor, & Pearce, 2000; Rose, 1961). Furthermore, heat stability can be improved by the addition of Ca−binding salts (to reduce Ca−ion activity) or phosphates (to enhance buffering capacity) (De Kort, *et, al.*, 2012).

Crowley, *et, al.*, (2014) studied the heat stability of MPC with 36.6% w/w (MPC 35) to 89.6% w/w (MPC 90) protein (dry matter) at 140 °C in the pH range 6.3-7.3. A decrease in heat stability with increasing protein content was reported at pH<6.8 and high Ca−ion activity, whereas at pH>6.8, the heat stability decline was countered by a reduced heat-induced κ-casein dissociation. Furthermore, heat stability can be restored by fortification with lactose and optimising the serum composition of skim milk. However, very limited information is available regarding the heat stability of reconstituted MPC powders with plant-based proteolytic hydrolysis treatment.

Although whey proteins are very soluble, this property is highly dependent on the pH of the system which creates problems during downstream processing and especially during thermal processing. Thus, a partial hydrolysis may improve stability of whey proteins by increasing their solubility and thus heat stability. Furthermore, these partial adjustments may improve other functional characteristics including foaming and emulsification. With an increase in the %DH, more proteins will be soluble due to the liberation of carboxylic and amine groups.

However, a study conducted by Mullally, *et, al.*, (1994) reported that the type of enzyme used is the main factor influencing solubility rather than %DH.

Most WPHs exhibit poor emulsification properties, however, this is dependent on both the %DH as well as molecular weight of the resultant hydrolysates. Emulsion activity (E_A) and emulsion stability (E_S) can be quantified and corelated with %DH. For example, in the case of WPC hydrolysis, a %DH <10% (by Neutrase®) resulted in improved E_A and %DH >10% (by Alcalase®) decreased E_A (Venter, McGill, & Lombard, 1989). Moreover, hydrolysates containing larger numbers of >2 kDa peptide fractions exhibit higher E_S (van der Ven, *et, al.*, 2001). Conversely, larger numbers of small peptides (<3 kDa) and free amino acids result in improved foam stability (Alder-Nissen, 1979). Hence, hydrolysates obtained with smaller M_w are more beneficial to the foaming properties of the resultant product.

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Literature Review Part B

Plant proteases and their application in dairy systems

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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.

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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).

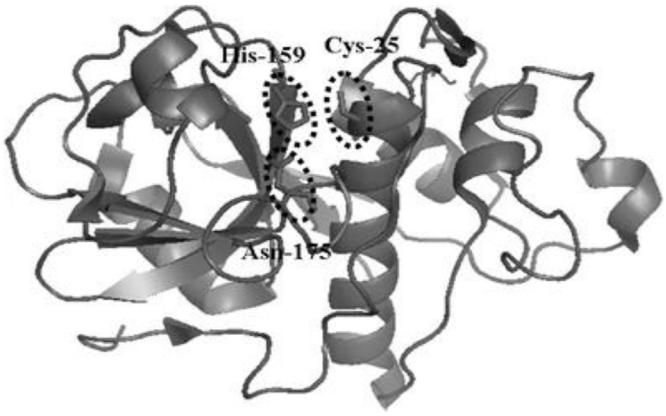

Fig. 1. Three-dimensional model of cysteine protease (papain), PDB code: 1PPN (adapted from Pickersgill, Harris, & Garman, 1992).

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.

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Chapter 3

Milk Protein Hydrolysis by Actinidin: Influence of Protein Source and Hydrolysis Conditions

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Short communication

Milk protein hydrolysis by actinidin: Influence of protein source and hydrolysis conditions

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ABSTRACT

The plant protease actinidin has been frequently used in the food industry, but its application in dairy systems remains largely unassessed. The aim of this research was to establish the effect of temperature (15–60 °C), time (0–5 h) and enzyme-to-substrate ratio on the actinidin-induced hydrolysis of proteins in whey protein isolate (WPI), whey protein concentrate (WPC) and milk protein concentrate (MPC), as monitored through the degree of hydrolysis (DH) and SDS-PAGE. The DH increased with increasing temperature and incubation time for all three protein sources. A lower E:S ratio resulted in a greater DH for WPC and WPI, but not for MPC. SDS-PAGE analysis revealed that actinidin mainly acted on α-lactalbumin and α_S-caseins in WPI and MPC, respectively.

1. Introduction

Actinidin (EC 3.4.22.14) is a 220 amino acid (23.5 kDa) cysteine protease isolated from kiwifruit and belonging to the cysteine peptide family C1, subfamily CA1 (papain family, clan CA (Baker, Boland, Calder, & Hardman, 1980). An extensive homology in the amino acid sequence, substrate specificity and tertiary structure of actinidin to papain has been found, a well-known protease from the same family (Baker et al., 1980). Actinidin has a wide substrate specificity, mostly cleaving peptide bonds at the carboxylic end of lysine residues. Actinidin is active over a broad range of pH (4–10) and temperature (15–60 °C) (Aspmo, Horn, & Eijsink, 2005; Zhu, Kaur, & Boland, 2018). Actinidin is used widely in meat process, but has received little attention to date in dairy applications. Previously, the use of actinidin has been only assessed to minimise antigenicity of β-lactoglobulin (β-LG) (Puglisi, Petrone, & Piero, 2012) as well at to minimise allergenicity and improve functionality of α-lactalbumin (α-LA) (Vázquez-Lara, Tello-Solís, Gómez-Ruiz, García-Garibay, & Rodríguez-Serrano, 2003).

Since actinidin has only been sporadically assessed for use in the dairy applications, the aim of the research was to establish the effects of proteolytic activity of actinidin on whey protein isolate (WPI), whey protein concentrate (WPC) and milk protein concentrate (MPC). The effect of incubation time, temperature and enzyme:substrate ratio on the degree of protein hydrolysis and protein hydrolysis patterns were assessed.

2. Materials and methods

2.1. Materials

WPI (90%, w/w, protein on dry matter), WPC (80%, w/w, protein on dry matter) and MPC (80%, w/w, protein on dry matter) were obtained from Fonterra Cooperative (Palmerston North, New Zealand). Actinidin KEP500 was sourced from kiwiEnzyme.com Ltd (Martinborough, New Zealand). The product was assayed at an actinidin activity of 521 units (U) g^{-1} dry matter based on the CBZ method (Heinrickson & Kézdy, 1976). The enzyme preparation contained 4.03% total solids out of which 90.0% on dry matter were proteins, as determined by the Kjeldahl method. Trinitrobenzenesulfonic acid (TNBS), sodium phosphate buffer (0.2125 M, pH 8.2) and sodium dodecyl sulphate (SDS) were of analytical grade and were obtained from Sigma–Aldrich Pvt Ltd (Castle Hill, Australia) and Merck KGa (Darmstadt, Germany). Simulated milk ultrafiltrate (SMUF) (pH 6.8) was used as a buffer for all samples and prepared as described by Rosmaninho and Melo (2006) and Ranadheera et al. (2019).

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2.2. Enzymatic hydrolysis of milk protein systems

Protein dispersions were by dispersing 5 g of protein powder into 95 g of SMUF and mixing continuously at 4 °C for 24 h for complete hydration (Liyanaarachchi & Vasiljevic, 2018). The protein dispersions were then equilibrated at 15, 35, 40, 55, 60 or 70 °C before addition of actinidin. The pH was not controlled, but monitored throughout hydrolysis using a portable pH meter (model 3110 SET2 ProfiLine, Xylem Analytics, Hemmant, QLD, Australia). Actinidin was added at two enzyme-to-substrate (E:S) ratios, i.e., 1.05 and 5.25 U g^{-1} of protein, and samples were subsequently incubated at the selected temperatures for up to 5 h with constant stirring in a shaking water bath (Grant Instruments Cambridge Ltd, Barrington, Cambridge, UK). An aliquot (1.0 mL) of samples was taken out at every 30 min and immediately mixed with 1.0 mL of 10% (w/v) SDS, added followed by heating at 85 °C for 5 min to terminate enzymatic reaction. All samples were subsequently stored at −20 °C prior to further testing.

2.3. Determination of the degree of hydrolysis of milk protein hydrolysates

Extent of cleavage of peptide bonds was determined by a spectrophotometric TNBS method of Adler-Nissen (1979) by measuring absorbance at 340 nm using a spectrophotometer (Biochrome Libra S12, Biochrom Ltd, Cambridge, UK). The experimental procedure also included samples without added enzyme as a control. Furthermore, fully hydrolysed samples were also included which was prepared by treating each substrate with 6 M HCl under reflux (method: 994.12, Official methods of analysis of AOAC International; AOAC, 1995). Hydrolysates were then filtered, neutralised and the absorbance was determined by the aforementioned TNBS method. The degree of hydrolysis was defined as a percentage of cleaved peptide bonds and calculated using the following equation (Adler-Nissen, 1986):

$$\%DH = \frac{h}{h\,tot} \times 100 \quad (1)$$

where h_{tot} is the total number of peptide bonds per protein equivalent obtained by given samples with chemical hydrolysis, and h is the number of hydrolysed bonds.

2.4. Sodium dodecyl sulphate polyacrylamide gel electrophoresis analysis of hydrolysed samples

Samples were analysed by SDS-PAGE to identify the patterns of proteolysis. The analysis was performed under reducing and non-reducing conditions with β-mercaptoethanol as the reducing reagent, as described previously (Bogahawaththa, Chandrapala, & Vasiljevic, 2017). Gels were scanned using the ChemiDoc imager (Chemidoc MP, Bio-Rad Laboratories, Hercules, CA, USA) and gels quantifications were performed in triplicate on all reducing gels using a software (Image Lab 6.0.1 @2017, Bio-Rad Laboratories Inc).

2.5. Statistical analysis

Hydrolysis experiments were replicated at least 3 times for all three substrates. The data was analysed as repeated in time measurements using a SAS software (v. 9.1) and the GLM protocol. The means were compared using the LSMEANS function with the PDIFF option and significance set at $P < 0.05$.

3. Results

3.1. Impact of process conditions on pH and degree of hydrolysis

The pH change during hydrolysis was monitored during incubation and the decrease in pH after 5 h, compared with the original sample is shown in Table 1. Two clear trends can be observed, i.e., (1) pH decrease increased with the rise in incubation temperature, and (2) pH decrease was notably greater for MPC in comparison with that of WPC and WPI, particularly at 55 and 60 °C. The greatest decline in pH of 0.57 was observed at 60 °C for the MPC sample.

DH for all samples increased with increasing incubation temperature (15–60 °C) (Supplementary material Figs. S1 and S2). WPI and MPC dispersions incubated at 70 °C with actinidin underwent a sol gel transition and could thus not be analysed for %DH. An increase in %DH with prolonged incubation time for all three substrates was noted and %DH did not plateau during the investigated time (5 h). The maximum %DH ranged from ~9 (for MPC) to ~16% (for WPI; Table 2). MPC had the lowest %DH, despite showing the greatest decrease in pH (Table 1). After 5 h of incubation at 60 °C, % DH of WPI and WPC significantly ($P < 0.05$) increased as an enzyme to substrate ratio changed from 1.05 to 5.25 U g^{-1} protein (Table 2). As opposed to these observations, %DH of MPC after 5 h of hydrolysis showed no difference between these two ratios resulting in a DH of 9.1% in both cases (Table 2).

3.2. Proteolysis patterns for actinidin-induced hydrolysis of WPI and MPC

To further elaborate on the actinidin-induced hydrolysis of milk proteins, the SDS-PAGE patterns of WPC and MPC samples incubated with actinidin were established. The data are shown in Supplementary material Fig. S3A–D. Protein patterns of WPC and MPC hydrolysed with actinidin at 15, 35 or 60 °C at an enzyme to substrate ratio of 5.25 or 1.05 U g^{-1} protein was compared. β-LG and α-LA bands were detected in all samples in addition to caseins (α_S-, β-, and κ-casein) detected in MPC. The band intensities lessened with increase in temperature and enzyme concentration (Table 3; Supplementary material Fig. S3). The enzyme action appeared mainly directed towards α-LA as compared with β-LG in the case of whey proteins. This was most apparent when the temperature increased to 60 °C, with ~37% and ~17% of β-LG and ~12% and ~4% of α-LA remaining after 5 h of incubation with the enzyme at 1.05 and 5.25 units g^{-1} protein, respectively (Table 2). Of the caseins, the α_S-CNs appeared more susceptible to hydrolysis

Table 1
The decrease in pH observed after 5 h incubation at 15–60 °C of dispersions (5% w/w) of milk protein concentration (MPC), whey protein isolate (WPI) and whey protein concentrate (WPC) with actinidin at an enzyme to substrate ratio (E:S) of 5.25 units g^{-1} protein or 1.05 units g^{-1} of protein.[a]

Temperature (°C)	E:S (units of actinidin activity g^{-1} protein)					
	5.25			1.05		
	MPC	WPI	WPC	MPC	WPI	WPC
15	0.01^{aA}	0.04^{aB}	0.02^{aA}	0.01^{aA}	0.02^{aA}	0.00^{aA}
35	0.18^{bD}	0.09^{bB}	0.07^{bA}	0.13^{bC}	0.08^{bA}	0.07^{bA}
40	0.18^{bC}	0.16^{cB}	0.19^{cC}	0.16^{cAB}	0.16^{cAB}	0.15^{cA}
55	0.32^{cB}	0.23^{dA}	0.23^{dA}	0.32^{dB}	0.22^{dA}	0.22^{dA}
60	0.57^{dC}	0.28^{eB}	0.28^{eB}	0.56^{eC}	0.27^{eB}	0.23^{dA}

[a] The pooled standard error of the mean of at least 3 independent observations was 0.005; lower and upper case superscript letters indicate significant difference ($P < 0.05$) within a column and a row, respectively.

Table 2
Degree of hydrolysis (DH) after incubation of 5% (w/w) dispersions of milk protein concentration (MPC), whey protein isolate (WPI) and whey protein concentrate (WPC) with actinidin at an enzyme to substrate ratio (E:S) of 5.25 units g^{-1} protein or 1.05 units g^{-1} of protein.[a]

E:S	Temperature of hydrolysis (°C)	DH (%)		
		Substrates		
		MPC	WPC	WPI
1:100	15	2.33^{fC}	5.12^{iB}	5.37^{iA}
	35	5.89^{dC}	8.06^{fB}	8.88^{fA}
	40	6.67^{cC}	10.90^{dB}	12.20^{dA}
	55	8.85^{bC}	14.10^{bB}	14.60^{bA}
	60	9.14^{aC}	15.00^{aB}	15.60^{aA}
1:500	15	2.25^{fC}	3.70^{jB}	4.68^{jA}
	35	5.39^{eC}	6.39^{hB}	7.20^{hA}
	40	6.43^{dC}	7.27^{gB}	7.41^{gA}
	55	6.13^{dC}	9.89^{eA}	9.62^{eB}
	60	9.10^{aC}	11.93^{cB}	12.64^{cA}

[a] The pooled standard error of the mean of at least 3 independent observations was 0.074; lower and upper case superscript letters indicate significant difference ($P < 0.05$) within a column and a row, respectively.

Table 3
Proportion of milk proteins (%) remaining after incubation of milk protein concentrate (MPC) (5%, w/w) and whey protein concentrate (WPC) (5%, w/w) with actinidin at an enzyme to substrate ratio (E:S) of 5.25 or 1.05 units g^{-1} protein at 60 °C for 5 h.[a]

Protein	E:S (units of actinidin activity g^{-1} protein)						SEM
	5.25			1.05			
	Temperature (°C)			Temperature (°C)			
	15	35	60	15	35	60	
MPC							
α_S-CN	48.80^{c}	18.20^{d}	9.30^{e}	59.40^{a}	54.63^{b}	9.27^{e}	0.50
β-CN	46.20^{c}	30.40^{d}	7.60^{f}	59.50^{a}	49.90^{b}	21.00^{e}	0.57
κ-CN	67.70^{b}	22.37^{c}	8.93^{e}	78.80^{a}	68.27^{b}	14.00^{d}	0.32
β-LG	54.10^{b}	47.20^{c}	36.80^{d}	57.10^{a}	53.50^{b}	36.30^{d}	0.37
α-LA	6.36^{c}	6.10^{c}	6.50^{c}	37.4^{a}	37.5^{a}	26.4^{b}	0.12
WPI							
β-LG	55.63^{b}	31.03^{e}	16.67^{f}	62.27^{a}	47.23^{c}	36.97^{d}	0.29
α-LA	21.36^{b}	8.33^{e}	4.33^{f}	22.76^{a}	16.27^{c}	11.67^{d}	0.11

[a] The proportion expressed is relative to the unhydrolysed control estimated from the reducing SDS-PAGE gels. SEM: pooled standard error of the mean of at least 3 independent observations; lower case superscript letters indicate significant difference ($P < 0.05$) within a row.

than β-CN and κ-CN, especially at the higher E:S ratio (Table 3; Supplementary material Fig. S3).

In MPC, β-LG again was not as much affected as α-LA after 5 h of incubation. Interestingly, while the proteins in MPC at 60 °C underwent little or no hydrolysis as shown by %DH at both E:S ratios, SDS patterns of MPC hydrolysis indicate overall disappearance of intact casein bands depending on temperature. Furthermore, larger aggregates can be observed on the top of the stacking non-reducing SDS-PAGE gel after hydrolysis at low temperature, which disappeared when a reducing agent was added (Supplementary material Fig. S3C,D). Obviously, the proteolysis led to creation of disulphide linked aggregates at this temperature, which were not present at higher temperatures. From these observations it is not clear whether the hydrolytic patterns are the same leading to creation of aggregated intermediates that a consequently cleaved or this intermediate step is absent at elevated temperatures. Also, it appears that individual peptides formed upon cleavage at higher temperature (60 °C) were likely smaller than the smallest milk protein (α-LA) as they eluted from the gel. This is indicated by the intensity of the protein bands in the reducing SDS-PAGE, which has not changed in comparison with the non-reducing SDS-PAGE.

4. Discussion

Four parameters (E:S ratio, incubation temperature, incubation time and protein substrate), were studied to determine the impact of each parameter on degree of hydrolysis of milk proteins by actinidin. Progressive increase in %DH with elevation of temperature and time indicates the availability of cleavage sites, leading to rise in number of free amino groups and smaller peptide chains after hydrolysis (Salwanee, Aida, Mamot, Maskat, & Ibrahim, 2013). Similar trend in %DH has been seen in previous studies where the thiol plant proteases bromelain and papain were used in various dairy systems, achieved a maximum %DH of ~20% and ~16% for skimmed goat milk, and ~23% and ~17% for skimmed cow milk, respectively (Shu et al., 2018). Our study simply confirmed previous reports, which assessed other plant derived proteases (Al-Shamsi, Mudgil, Hassan, & Maqsood, 2018; Kumar, Chatli, Singh, Mehta, & Kumar, 2016), highlighting time/temperature relation of proteolytic activity of these enzymes with the extent of hydrolysis. Their activity was also dependant on a number of available cleavage sites, which related to a type of substrate (Hashim, Maskat, Wan mustapha, & Mamot, 2010). Salwanee et al. (2013) reported similar observations when %DH increased when alcalase concentration increased from 1% to 1.5%. The current study also indicates that the E:S ratio needs be optimised to achieve a %DH maximum in shortest time possible under defined conditions. For actinidin, this ratio clearly depended on the type of the substrate as changing E:S ratio for MPC had no impact on %DH (Supplementary material Figs. S1 and S2), which was lower in comparison with that of WPC or WPI.

While whey proteins appeared to be preferred substrate for this actinidin, proteolytic activity on the caseins was also obvious from the SDS-PAGE gels (Fig. S3C,D). Al-Shamsi et al. (2018) reported similar findings when camel milk proteins were hydrolysed with the plant-derived cysteine proteases papain and bromelain. Their SDS-PAGE analysis showed total disappearance of casein bands while %DH was just under 25% and negligible band intensity at around 10% DH. Moreover, in the same study, hydrolysis using alcalase, a serine protease, resulted in a limited DH of ~7% but no residual casein bands were observed in the associated SDS-PAGE gels after 2 h of incubation (Al-Shamsi et al., 2018). It is worth noting that it requires only a single bond cleavage of an individual casein molecule to disappear from the gel. Based on the results a greater %DH may indicate that the caseins were further hydrolysed into several polypeptide chains. Greater hydrolysis of α-LA than that of β-LG could likely be attributed to structural differences between these two proteins that hindered access of the enzyme to active sites rather than in a number of active sites as β-LG contains more lysine residues than α-LA (Brew, 2013; Sawyer, 2013).

In the current study, uncontrolled but monitored pH (Table 1) is an important parameter as there is less or no demand to control pH at industrial level to simplify the process, eliminate the chances of contamination and produce a final product without additives (Le Maux, Nongonierma, Barre, & FitzGerald, 2016). A study conducted by Fernández and Kelly (2016) on whey proteins with Protamex® resulted in a greater DH% at uncontrolled pH as compared with that obtained by a pH stat method. During hydrolysis of proteins, peptide bonds are cleaved and carboxyl and amino groups are released. At neutral pH, carboxyl groups are completely deionised, which initiates proton exchange between carboxyl and amino groups (Márquez & Vázquez, 1999). This consequently results in a decrease in the pH of the reaction mixture. This impacts not only the enzyme but also the substrate, especially the MPC, as the casein micelle would most likely change. Whey proteins, on the other hand, would be less affected by this change. Le Maux et al. (2016) showed that physiochemical properties of whey proteins

hydrolysates obtained by controlled and uncontrolled pH did not differ substantially. Hydrolysis indicated that actinidin has potential to act as or in replacement of other plant proteases where partial hydrolysis is required, however, its further assessment in improvement of physical or biological functionality is required.

5. Conclusion

Actinidin appears to prefer whey proteins over caseins as its substrate. The extent of hydrolysis established by %DH was clearly temperature dependant and peaked at 60 °C. In case of whey proteins upon addition of the greater enzyme activity (5.21 units g^{-1} protein), the DH increased significantly. The extent of MPC hydrolysis, however, was not affected by changing enzyme concentration. The PAGE analysis revealed α-LA as a preferential substrate fraction in case of whey proteins and α_S-casein in case of MPC. The pH decline was substrate and temperature dependant with a maximum difference of about 0.57 achieved during MPC hydrolysis at a E:S ratio of 5.25 units g^{-1} protein at 60 °C. Overall, actinidin exerted an appreciable activity towards dairy proteins as substrates. Its application should be further assessed in products where limited hydrolysis is needed such as modulation of a specific functionality or allergenicity.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2021.105029.

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3.2 Supplementary material

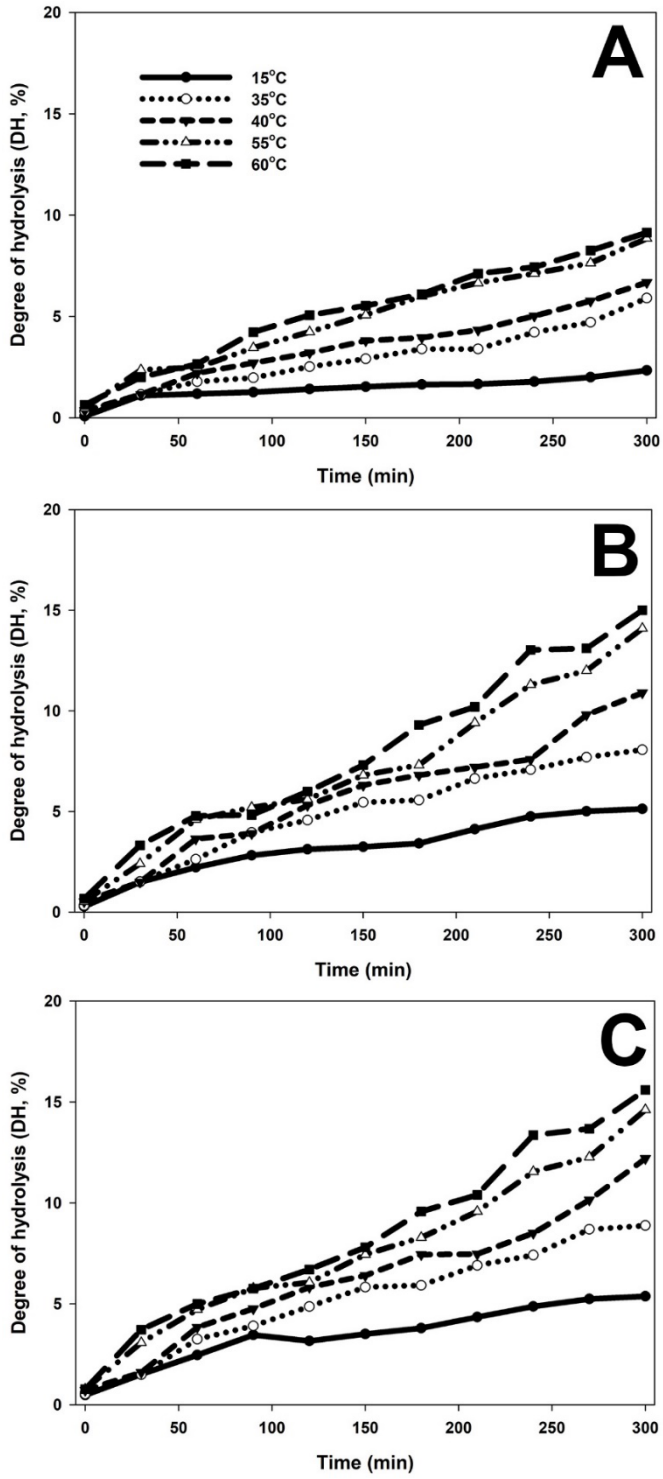

Fig. S1. Degree of hydrolysis after incubation of 5% (w/w) dispersions of MPC (A), WPC (B) and WPI (C) with actinidin at an enzyme to substrate ratio of 5.25 units of actinidin activity g^{-1} protein for up to 5 h at 15–60 °C.

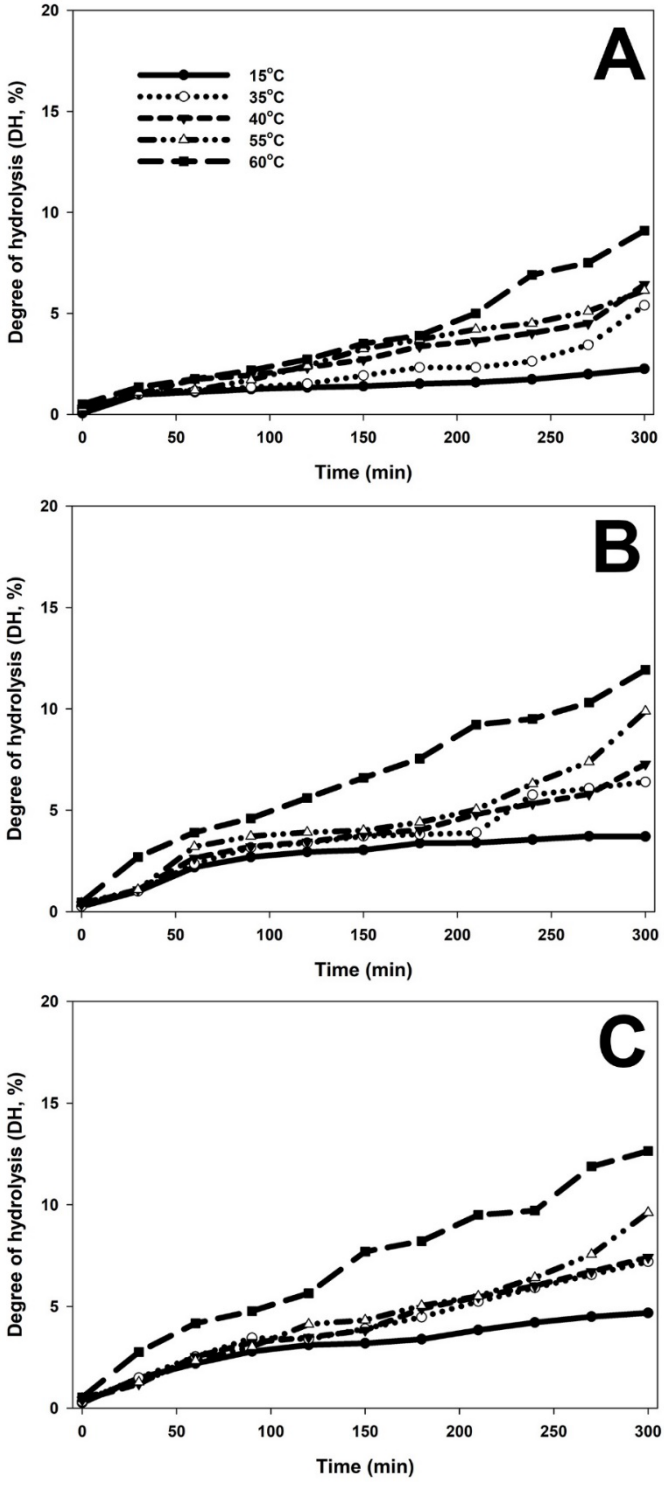

Fig. S2. Degree of hydrolysis after incubation of 5% (w/w) dispersions of MPC (A), WPC (B) and WPI (C) with actinidin at an enzyme to substrate ratio of 1.05 units of actinidin activity g^{-1} protein for up to 5 h at 15–60 °C.

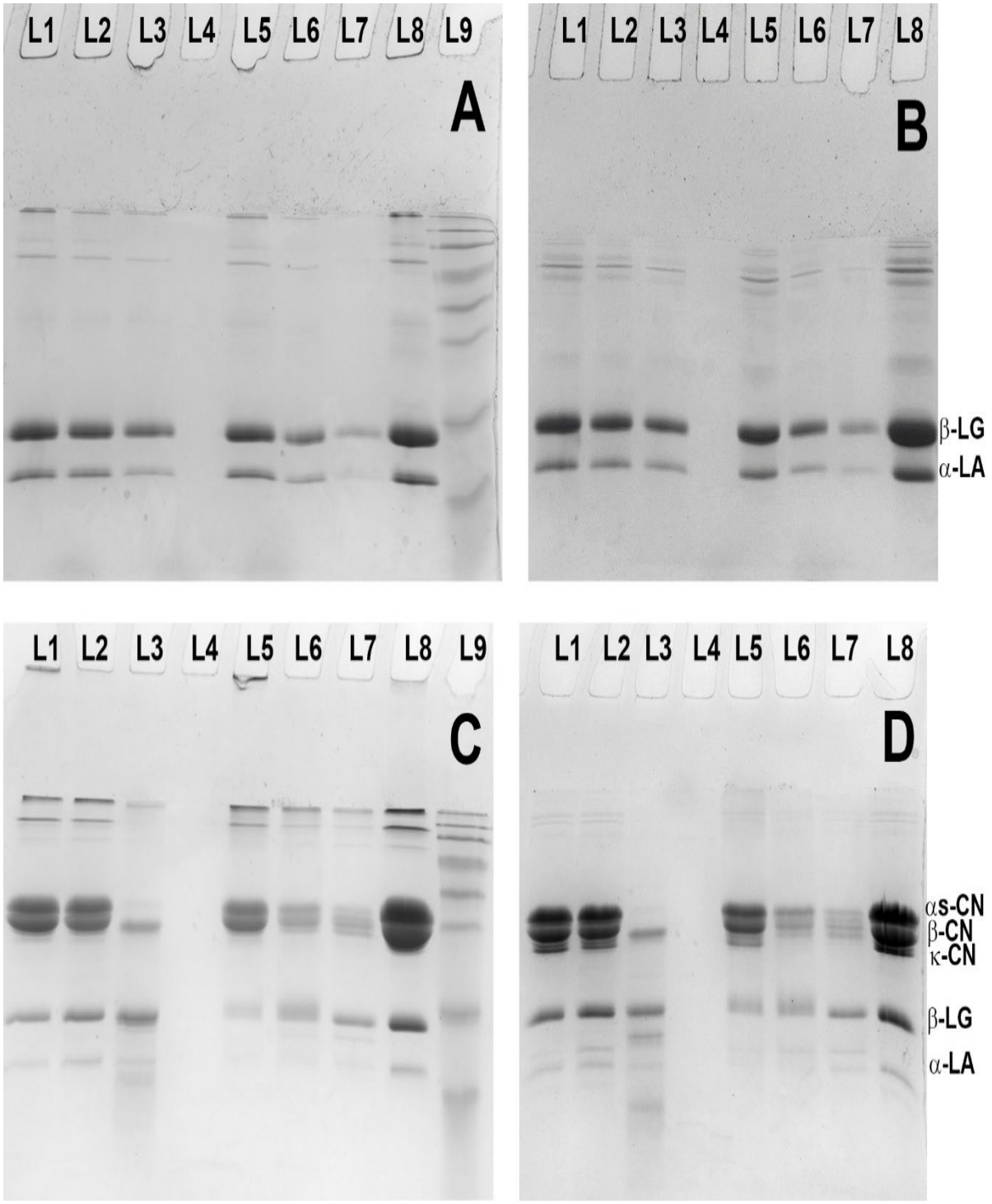

Fig. S3. Non-reducing (A, C) and reducing (B, D) SDS-PAGE electrophoretograms of whey protein isolate (A, B) and milk protein concentrate (C, D) incubated with actinidin treatments for 5 h at an enzyme substrate rate of 1.05 units of actinidin activity g^{-1} protein (L1, L2, L3) or 5.25 units of actinidin activity g^{-1} protein (L5, L6, L7) at 15 (L1, L5), 35 (L2, L6) or 60 °C (L3, L7). L4, enzyme; L8, control untreated; L9, molecular mass marker.

Chapter 4

Actinidin-Induced Hydrolysis of Milk Proteins: Effect on Antigenicity

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This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

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I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

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Name(s) of Co-Author(s)	Contribution (%)	Nature of Contribution	Signature	Date
Surjit Kaur	80 %	Designing and conducting the experiment, data collection, statistical analysis and manuscript preparation	[redacted]	7/2/2024
Prof Todor Vasiljevic	10 %	Desinning the experiment, statistical analysis, manuscript editing and submission to the Journal		07/02/2024
Prof Thom Huppertz	10 %	Designing the experiment and manuscript editing		7/2/2024

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Actinidin-induced hydrolysis of milk proteins: Effect on antigenicity

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ABSTRACT

Actinidin was used to hydrolyse proteins in whey protein isolate (WPI) and milk protein concentrate (MPC) to reduce immunoreactivity of β-lactoglobulin (β-LG) and αs_1-casein (αs_1-CN). Samples were incubated at an enzyme to substrate ratio of 1:100 (5.21 units of actinidin activity g^{-1} of protein) at 10 or 60 °C for up to 31 or 5 h, respectively. Protein hydrolysis was determined by the degree of hydrolysis and SDS-PAGE. Antigenicity of hydrolysates was determined by β-LG and αs_1-CN antibody-binding capacity using enzyme-linked immunosorbent assay (ELISA) quantification kits. ELISA showed significant reduction of antigenicity of β-LG and αs_1-CN with higher degree of hydrolysis (DH) by actinidin. At 60 °C, hydrolysis for 5 h resulted in antigenicity reduction of ~43% for β-LG and ~48% for αs_1-CN in MPC and ~54% for β-LG in WPI. Hydrolysis at 10 °C for 31 h also resulted in decrease in antigenicity in MPC for β-LG and αs_1-CN by ~39 and 42% respectively, but only 14% for β-LG in WPI. Treatment with actinidin can reduce the antigenicity by modification of protein conformation and cleavage and masking of epitopes of β-LG and αs_1-CN.

1. Introduction

Actinidin (EC 3.4.22.14) is a thiol plant protease obtained from kiwifruit with a molecular weight of 23.5 kDa and contains 220 amino acids (Boland & Singh, 2013). The enzyme was first described by Arcus (1959) but, thus far, actinidin has been sporadically assessed for the applications in the dairy industry. Recently, we evaluated the performance of actinidin on selected milk protein ingredients (Kaur, Huppertz, & Vasiljevic, 2021), noting that the enzyme hydrolysed preferentially whey proteins, especially α-lactalbumin (α-LA). The activity was also temperature-dependant with the greatest degree of hydrolysis (%DH) of ~16% being obtained at 60 °C after 5 h. The enzyme lost activity above 65 °C (Kaur et al., 2021). This indicated that actinidin could potentially be used for modifications of dairy proteins, e.g., for improvements of certain functional properties or minimizing antigenicity.

The allergenic nature of milk proteins can result in some limitations to its application in some foods (Bogahawaththa, Chandrapala, & Vasiljevic, 2017). Cow milk protein allergy (CMPA) is the most prevalent food allergy among infants (2–6%) in Western countries and is both IgE and IgG mediated (Bartuzi, Cocco, Muraro, & Nowak-Węgrzyn, 2017; Hochwallner, Schulmeister, Swoboda, Spitzauer, & Valenta, 2014). The allergenic nature of milk proteins, such as the caseins (CN), β-lactoglobulin (β-LG), bovine serum albumin (BSA) and α-lactalbumin (α-LA), is due to the presence of conformational and sequential epitopes. Milk proteins exert their allergenicity/antigenicity by binding with IgE and/or IgG, which ultimately may induce an allergy reaction in humans (Bogahawaththa, Buckow, Chandrapala, & Vasiljevic, 2018).

While avoidance of foods containing milk proteins has been one approach in the management of CMPA, this is not a recommended practice due to importance of these proteins as well as other dairy components, e.g., minerals and other micronutrients, in the diet. Therefore, establishing approaches to diminish allergenicity of these proteins is of great importance. Molecular structure of milk proteins can be modified by several approaches, including heat treatment (Bogahawaththa et al., 2017), fermentation (Jia et al., 2021), pressurization, non-enzymatic glycosylation (Bu, Luo, Chen, Liu, & Zhu, 2013) and enzymes (Wróblewska & Troszyńska, 2005). Many studies have shown that caseins, unlike whey proteins, maintain their allergenicity even after heat treatment (Castillo & Cassola, 2017; Restani, Ballabio, Di Lorenzo, Tripodi, & Fiocchi, 2004. Extensively (eHF) or partially hydrolysed (pHF) infant formula are recommended as first alternative in CMPA children. Differences exist between these formulas, especially in

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the amount of β-lactoglobulin since its level in pHF is substantially greater than that in eHF (Exl & Fritsche, 2001). Modern hydrolysed infant formulas differ due to protein source, a degree and type of hydrolysis and profiles of released peptides, all of which are enzyme dependant in addition to other pre- and post-processing methods (Exl & Fritsche, 2001). The main focus of research activities in this area has been placed at diminishing allergenicity of proteins while retaining other properties most notably sensory acceptability. While numerous studies have been conducted and knowledge in this area has been expanded, the research is still ongoing on improving these formulas (Davisse-Paturet et al., 2019; Virtanen et al., 2021). Thus, further studies are needed with a multitude of objectives including enzyme selection and establishment of processing conditions that would result in hypoallergenic hydrolysates. While some plant proteases, including papain, were recently assessed for reduction of cow milk antigenicity (Liang et al., 2020), actinidin has not been evaluated so far.

The focus of this study thus was to examine the effect of actinidin-induced protein hydrolysis on the antigenicity of αs_1-CN and β-LG. The focus was on β-LG and α_{S1}-CN, since more than 50% of human population is sensitised mainly by these proteins (Rezvan Asghar, Ahmad, & Reihane, 2018). β-LG and αs_1-CN appear to be the main allergens (Schulmeister et al., 2009; Wal, 2004) and studies conducted on a large group of infants showed the allergenic nature of cow milk protein was due to mainly these two fractions (Schulmeister et al., 2009; Wal, 2004). αs_1-CN is a single chain phosphoprotein, comprised of 199 amino acids, and has the highest potential for allergenicity due to presence at least seven epitopes (Cerecedo et al., 2008; Cong, Yi, Qing, & Li, 2013). β-LG is the most abundant whey protein with 162 amino acid residues and molecular weight of ~18.3 kDa. The structure of β-LG has 9 strands, A to I, with the first two described as the main regions that possess allergenic potential (Maier, Okun, Pittner, & Lindner, 2006). Since allergenic properties of these proteins can be reduced by hydrolysis into peptides and free amino acids (Hajihashemi, Nasirpour, Scher, & Desobry, 2014), we hypothethised that using actinidin under commercially relevant conditions may achieve appreciable antigenicity reduction. As allergenicity can be due to two types of epitopes, the research question was whether conformational changes could be related to changes in the antigenicity. In this work, two commercially important dairy products - milk protein concentrate (MPC) and whey protein isolate (WPI) in solutions – were hydrolysed by actinidin at two temperatures (10 and 60 °C) and its effect on antigenicity of αs_1-CN and β-LG was studied.

2. Materials and methods

2.1. Materials

WPI (90%, w/w, protein) and MPC (80%, w/w, protein) were obtained from Fonterra Co-operative (Palmerston North, New Zealand). Actinidin, with an activity of 521 units/g dry matter, based on the CBZ method (Heinrickson & Kézdy, 1976), was kindly donated by kiwiEnzyme.com Ltd (Martinborough, New Zealand). All other chemicals were of analytical grade and were obtained from Sigma-Aldrich Pvt Ltd (Castle Hill, NSW, Australia) and Merck KGa (Darmstadt, Germany). A bovine β-LG enzyme-linked immunosorbent assay (ELISA) quantification kit was obtained from Bethyl Laboratories Inc. (Montgomery, TX, USA) and the bovine αs_1-CN ELISA quantification kit was purchased from SAB (College Park, MD, USA).

2.2. Sample preparation and enzymatic hydrolysis

Dispersions (5%, w/w) of WPI and MPC in simulated milk ultrafiltrate (SMUF) were prepared as described previously (Kaur et al., 2021). Hydrolysis of WPI and MPC was performed with actinidin at the enzyme to substrate ratio (E:S) of 1:100 (5.21 units of actinidin activity/g of protein) at uncontrolled pH at 60 °C for 0–5 h and at 10 °C for 0–31 h in a shaking water bath followed by enzyme inactivation and sample storage for further analysis as described previously (Kaur et al., 2021). This specific temperature (60 °C) was chosen as the degree of hydrolysis (% DH) was the greatest, at ~16% after 5 h at this temperature (Kaur et al., 2021). The enzyme appeared to be denatured above 65 °C. Aliquot samples (1.0 mL) were taken out during hydrolysis at above mentioned time intervals and then 1.0 mL of 5% (w/v) SDS was added followed by heating at 80 °C for 5 min to terminate enzymatic reaction. Such treated samples were stored at −20 °C for further analysis. For antigenicity testing, 1.0 mL of a 5% SDS solution at 80 °C was added to 1.0 mL of sample to stop enzymatic activity. The original samples without added enzyme were treated under the same conditions and served as controls.

2.3. Analytical methods

2.3.1. Determination of the degree of hydrolysis

The degree of hydrolysis (%DH) was determined using the method of Adler-Nissen (1979) by determination of free amino groups using the trinitrobenzenesulfonic acid (TNBS) procedure as described previously (Kaur et al., 2021). %DH was calculated on a basis of the complete hydrolysis (Kaur et al., 2021) as:

$$\%DH = \frac{h}{h_{tot}} * 100 \quad (1)$$

where h_{tot} is the total number of peptide bonds per protein equivalent obtained by given samples with chemical hydrolysis, and h is the number of hydrolysed bonds following enzymatic hydrolysis.

2.3.2. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Samples were analysed by SDS-PAGE under reducing conditions using β-mercaptoethanol, as described by Bogahawaththa et al. (2017), to monitor hydrolysis of individual milk proteins. The gels were scanned using a ChemiDoc imager (Chemidoc MP, Bio-Rad Laboratories, Hercules, CA, USA). The gel quantification was achieved by a software (Image Lab 6.0.1 @2017, Bio-Rad Laboratories Inc). All determinations were performed in triplicate.

2.3.3. Fourier transform infrared (FTIR) spectroscopy

Immediately after sampling, all samples, including controls, were analysed using a FTIR spectrometer (Frontier, PerkinElmer, Boston, MA, USA). After using SMUF for a baseline correction, the samples were scanned in the 4000–600 cm^{-1} range with 16 scans per spectra at a resolution of 4 cm^{-1}. The spectra were resolved with Origin Pro 2020 software (Origin Lab Corporation, Northampton, MA, USA). First, the peak deconvolution was performed using the Fourier self-deconvolution (FSD) function within broad amid I region of 1600–1700 cm^{-1}. Baseline correction was performed followed by peak finding and peak fitting with aid of the Gaussian function with required level of iterations. The following four structural motifs were assessed for changes: β-sheets (1637-1610 cm^{-1} and 1696-1680 cm^{-1}), random coils (1648-1638 cm^{-1}), α-helices (1660-1650 cm^{-1}), and β-turns (1679-1667 cm^{-1}) (Grewal, Huppertz, & Vasiljevic, 2018).

2.3.4. Determination of antigenicity of β-LG and αs_1-CN

Samples were assessed for antigenicity using the bovine β-LG (for MPC and WPI) and αs_1-CN (for MPC only) ELISA quantitation kits according to the manufacturers' instructions, as previously described (Bogahawaththa, Chandrapala, & Vasiljevic, 2019; Kleber, Krause, Illgner, & Hinrichs, 2004; Rahaman, Vasiljevic, & Ramchandran, 2017). In brief, 96-well microtiter plates were coated with capture antibody (polyclonal rabbit IgG) that were raised against native bovine β-LG. On the other hand, bovine αs_1-CN detection kit was received with an already coated plate.

Samples and standards were serial diluted to maintain β-LG and αs_1-CN concentration limits within ranges of 1.95–125 ng/ml and 0.78–50

ng/ml, respectively. Exactly 100 μL of standards and diluted samples were added to a well separately and incubated for 1 and 2 h at room temperature and 37 °C for β-LG and αs_1-CN, respectively. After addition of β-LG-detecting antibody and tetramethylbenzidine (TMB) substrate, (or Detection Reagent A followed by Detection Reagent B in case of αs_1-CN substrate solution, the plates were kept in the dark for 15 min for colour development. The reaction was stopped by adding sulphuric acid and the absorbance was measured at 450 nm using a microplate reader (iMark, Bio-Rad Laboratories, Gladesville, NSW, Australia). A standard curve was constructed with each standard absorbance against its corresponding concentration (ng/ml). From the curve, the antigenicity (mg/ml) of β-LG and αs_1-CN was quantified. The proportion (%) of residual antigenicity (RA%) was expressive in relation to appropriate control sample:

$$Residual\ antigenicity\ (\%) = \frac{Antigenicity\ of\ hydrolysate}{Antigenicity\ of\ control\ sample} \cdot 100\% \quad (2)$$

2.4. Statistical analysis

All experiments were replicated 3 times on separate occasions for both substrates and the data were expressed as the mean ± SD of three independent assays. The data was analysed as repeated in time measurements using a SAS software (v. 9.1) and the GLM protocol. The means were compared using the LSMEANS function with the PDIFF option and significance level was set at $p < 0.05$. Several parameters were correlated including antigenicity reduction with residual intact β-LG or residual intact α_{s1}-CN, antigenicity with FTIR and correlation of residual intact proteins (SDS-PAGE summed scores based on proteins composition) with DH. These parameters were analysed by Pearson's correlation coefficient (r) that measures the degree of association between the variables using the IBM® SPSS® statistics software (student v.).

3. Results

3.1. Impact of temperature on hydrolysis and its correlation with proteolytic pattern and secondary structure of proteins

In line with previously reported data (Kaur et al., 2021), temperature had a substantial effect on the extent of protein hydrolysis by actinidin, with more extensive hydrolysis, determined as %DH (Fig. 1) and SDS-PAGE (Table 1 and Fig. 2) observed after hydrolysis at 60 °C than at 10 °C. The %DH obtained at 10 °C appeared in line with those reported

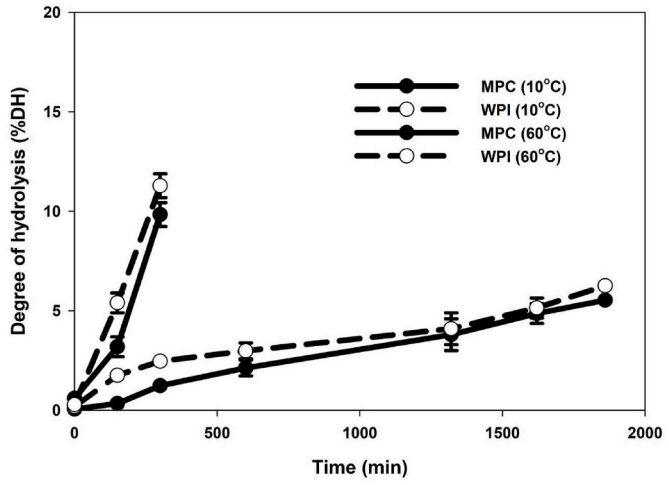

Fig. 1. Degree of hydrolysis (%DH) of 5% MPC and WPI solutions after incubation with actinidin at a 1:100 enzyme to substrate ratio for up to 31 h at 10 °C and up to 5 h at 60 °C.

Table 1
Proportion (%) of intact milk proteins remaining (relative to control) after incubation of 5% MPC and WPI solutions with actinidin at a 1:100 enzyme to substrate ratio for up to 31 h at 10 °C and up to 5 h at 60 °C.

Protein	MPC/10 °C				MPC/60 °C		
	Time of hydrolysis (h)						
	0.16	2.5	10	31	0.16	2.5	5
αs_2-CN	63.2^{aF}	55.0^{bD}	27.1^{eF}	19.5^{gF}	51.9^{cF}	47.6^{dC}	21.1^{fE}
αs_1-CN	74.1^{cC}	65.1^{dC}	35.7^{fC}	32.7^{gA}	94.8^{aA}	77.3^{bA}	53.9^{eA}
β-CN	65.6^{aE}	45.0^{bG}	31.1^{eD}	24.0^{gC}	38.2^{cG}	33.7^{dE}	30.4^{fB}
κ-CN	65.9^{aE}	48.1^{cE}	17.7^{eG}	10.4^{fG}	62.7^{bC}	28.8^{dG}	17.6^{eF}
β-LG	73.7^{bD}	46.2^{dF}	30.4^{eE}	22.8^{gE}	88.8^{aB}	59.5^{cB}	28.6^{fC}
α-LA	55.8^{aG}	0.0^{cH}	0.0^{cH}	0.0^{cH}	38.7^{bG}	0.0^{cH}	0.0^{cG}
	WPI/10 °C				WPI/60 °C		
	0.16	2.5	10	31	0.16	2.5	5
β-LG	84.2^{aB}	84.1^{aA}	46.4^{cA}	23.8^{fD}	60.9^{bD}	40.7^{dD}	27.8^{eD}
α-LA	92.0^{aA}	74.7^{bB}	42.8^{dB}	27.6^{fB}	58.9^{cE}	31.7^{eF}	21.1^{gE}

The pooled standard error of the mean (SEM) of at least 3 independent observations for hydrolysis of MPC and WPI and was 1.32 and 0.28, respectively; lower- and upper-case superscript letters indicate significant difference ($P < 0.05$) within a row and a column, respectively.

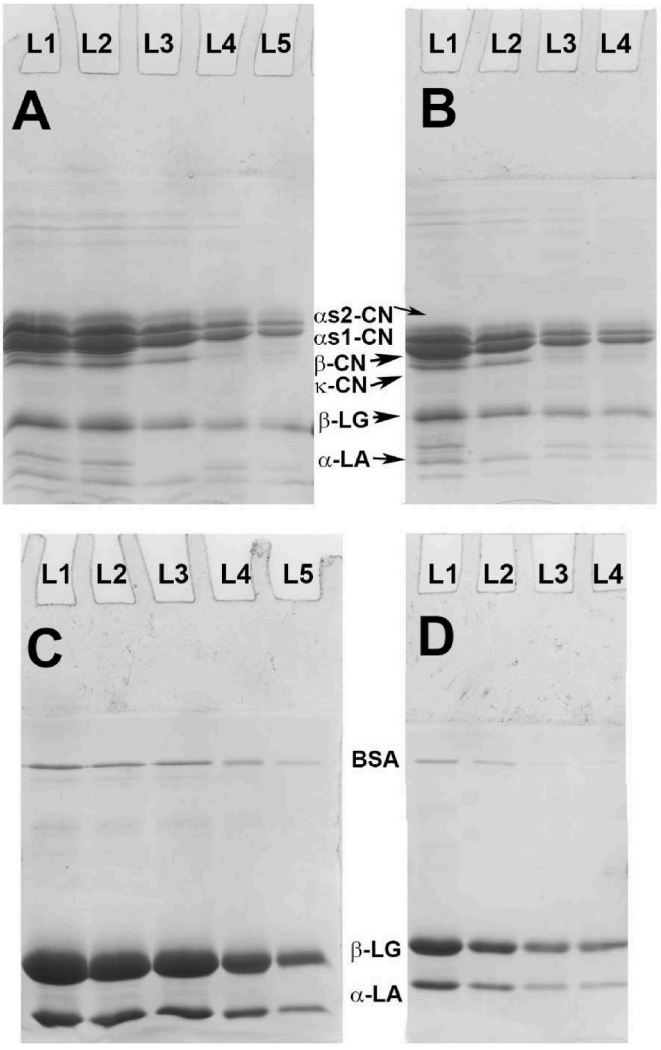

Fig. 2. Reducing SDS- PAGE patterns of MPC (A,B) and WPI (C,D) after incubation with actinidin at a 1:100 enzyme to substrate ratio at 10 °C (A,C) or 60 °C (B,D). L 1 = Control; 2 = 0.16 h; 3 = 2.5 h; 4 = 10 h; 5 = 31 h.

by Liang et al. (2020), who used papain to hydrolyse cow's milk at 20 °C for 2 h. The increase in %DH after 5 h at 60 °C was approximately two times higher than that after 31 h at 10 °C for both MPC and WPI (Fig. 1). Interestingly, however, the proportion of residual intact milk proteins did not show this trend (Table 1). In fact, residual intact proteins were higher after 5 h at 60 °C than after 31 h at 10 °C for all proteins except α-LA in MPC, and were higher for β-Lg but lower for α-LA in WPI (Table 1). The difference between hydrolysis monitored by %DH and SDS-PAGE is that the former considers every peptide bond hydrolysed in a protein, whereas the latter is based on the first peptide bond hydrolysed in a protein. The higher %DH at 60 °C than at 10 °C (Fig. 1) thus suggests more extensive hydrolysis of (poly)peptides from proteins that had undergone initial hydrolysis, rather than more protein molecules being subjected to hydrolysis. This indicates that the initial hydrolysis of the intact protein, rather than the subsequent further hydrolysis of the formed peptides, is rate-determining.

In addition to %DH and SDS-PAGE, protein hydrolysis was also monitored by FTIR (Table A1). In general, though, only limited changes were observed in structural elements of the milk proteins and clear trends as a function of incubation time, temperature and protein source were not readily apparent (Table A1). This could be linked to fact that % DH was comparatively low (Fig. 1) and that although the majority of the proteins had been hydrolysed at end of the incubation at both temperatures (Table 1), the initial peptide bonds hydrolysed in a protein are typically those readily accessible and not those involved in structural elements in the proteins (Buczek, Krowarsch, & Otlewski, 2002; Siezen & Hoenders, 1979).

3.2. Influence of actinidin hydrolysis on antigenicity in MPC and WPI

In addition to protein hydrolysis, changes in β-LG and α_{S1}-CN antigenicity were studied, the results of which are shown in Table 2. Reductions in antigenicity of β-LG were observed in both MPC and WPI and for MPC reductions in α_{s1}-CN were observed, the extent of which increased with increasing incubation time. For β-Lg in MPC, both incubation for 5 h at 60 °C and 31 h at 10 °C resulted in a decrease in antigenicity by ~40%, whereas in WPI, a notably larger decrease in β-Lg antigenicity after 5 h at 60 °C (>50% reduction) compared to 31 h at 10 °C (<15% reduction) despite both having a comparable level of residual intact β-Lg (Table 1). It is also worth noting that the reduction in residual intact β-Lg was larger than that in residual β-Lg antigenicity. In other words, the hydrolysis of a single peptide bond in β-Lg is insufficient to reduce antigenicity. For α_{S1}-CN in MPC, for both incubation at 10 and 60 °C comparable levels of residual intact α_{S1}-CN (Table 1) and residual α_{S1}-CN antigenicity (Table 2) was found, with again reductions in intact α_{S1}-CN being larger than those in α_{s1}-CN antigenicity.

4. Discussion

The allergenic nature of milk proteins limits their use in food products for some parts of the population. Allergenicity is due to the presence of specific amino acid sequences in the primary and secondary structures of proteins/peptides. Epitopes can be linear, conformational or overlapping, but conformational epitopes appear the cause of most allergies (Cong et al., 2013). Proteolysis can disrupt linear and conformational epitopes (Bu et al., 2013) and is one of the approaches to reduce antigenicity of milk allergens (Alting, Meijer, & van Beresteijn, 1998). Furthermore, proteolysis may also lead to formation of new epitopes or appearance of hidden epitopes (Bu et al., 2013). In this study, actinidin, as an unexplored plant protease, was assessed for its impact on milk proteins. Our previous study (Kaur et al., 2021) showed that actinidin can act over a broad range of temperatures, thus the extremes (10 and 60 °C) were applied in the current study. Antigenicity could be related to the content of intact proteins as a highly positive relationship was observed (Table A2). Hydrolysis of substrates containing α_{S1}-CN and β-Lg fractions by actinidin led to a significant reduction in antigenicity of these milk proteins. The ability of actinidin to reduce immunoreactivity of MPs by its hydrolytic action was temperature and time dependent and a correlation could be clearly observed.

Substantial differences between β-Lg and α_{S1}-CN in binding patterns of IgG antibodies to epitopes reflect their differences in the structure, where β-Lg was structurally more stable and compact due to possession of two disulphide bonds in contrast to α_{S1}-CN which is regarded as flexible and unstructured protein (Bu et al., 2013). Actinidin mostly cleaves amino acids present on hydrophobic sites of proteins such as Leu, Val or Phe (Boland & Singh, 2013). For αs_1-CN, the epitopes that could contain a possible cleavage sites include f(21–35) and f(161–175) (Cong et al., 2013). For β-Lg, the main allergenic epitopes include f (41–60), f(102–124) and f(149–162) (Bogahawaththa et al., 2017), all of which contain at least one of three possible cleavage sites. Cleavage of these epitopes may lead to a reduction in the antigenicity. These epitopes are also part of defined structural elements of β-Lg; for example, f (41–60) is part of β-strands and present at the surface of the molecule (Fox, 2003), therefore it was expected to see some structural changes upon their cleavage, which was not clearly demonstrated by the FTIR analysis. To further elaborate on the specificity, using a well-defined system could be considered.

A substantial antigenicity reduction was obtained even at the low temperature (Table 2), and a positive correlation was cursorily observed between remaining antigenicity and residual intact α_{S1}-CN or β-Lg (Table A2) which could likely be attributed to the effect of temperature. α_{s1}-CN has only a small amount of a defined secondary structure (α-helix, β-sheets, β-turn) and lacks disulphide bonds, thus its spatial conformation is stabilised by mainly hydrophobic interactions

Table 2
The antigenicity of β-LG and αs_1-CN (mg/ml) after incubation of 5% MPC and WPI solutions with actinidin at a 1:100 enzyme to substrate ratio for up to 31 h at 10 °C and up to 5 h at 60 °C.

β-LG								
Sample	0	Hydrolysis time (h)						
		0.16	2.5	5	10	22	27	31
MPC/10 °C	2.5^{aE}	2.5^{aE}	2.2^{bE}	2.1^{bE}	1.8^{cC}	1.7^{cC}	1.6^{dC}	1.5^{dC}
MPC/60 °C	2.1^{aF}	2.0^{aF}	1.5^{bF}	1.2^{cF}				
WPI/10 °C	14.8^{aC}	14.8^{aC}	14.8^{aA}	14.5^{bA}	13.9^{cA}	12.8^{dA}	12.8^{dA}	12.8^{dA}
WPI/60 °C	17.3^{aA}	15.8^{bB}	11.0^{cC}	8.0^{dC}				
αs_1-CN								
Sample	0	Hydrolysis time (h)						
		0.16	2.5	5	10	22	27	31r
MPC/10 °C	16.8^{aB}	16.5^{bA}	12.2^{cB}	12.0^{dB}	10.7^{eB}	10.5^{fB}	10.5^{fB}	9.9^{gB}
MPC/60 °C	12.9^{aD}	10.3^{bD}	7.8^{cD}	6.7^{dD}				

The pooled standard error of the mean (SEM) of at least 3 independent observations was 0.036; lower- and upper-case superscript letters indicate significant difference ($P < 0.05$) within a row and a column, respectively.

(Kumosinski, Brown, & Farrell, 1991). Since hydrophobic interactions are limited at low temperature, that would likely lead to more random structures and changes in conformational epitopes. In addition, low temperature also alters casein interactions and diffusion of individual caseins out of the micelle making them more accessible to the enzyme but also creating rather a more porous micellar structure that could be accessed by the enzyme (Bhat, Dar, & Singh, 2016).

Furthermore, α_{S1}-CN antigenicity reduction was greater in the early stages of proteolysis similar to observations obtained in another study (Dąbrowska et al., 2020), in which hydrolysis of α_{S1}-CN by *Yarrowia lipolytica* for 1 h resulted in antigenicity reduction by $\leq$ 30%. Further increase in %DH during 5 h hydrolysis resulted in a negligible reduction in α_{S1}-CN antigenicity (Dąbrowska et al., 2020). Results from the SDS-PAGE (Fig. 2, Table 1) obtained in our study show that α_{S1}-CN band intensity diminished with the extension of hydrolysis time at low temperature, which could also be related to decline in antigenicity. Also, a high positive correlation coefficient was noted when residual α_{S1}-CN was correlated with the remaining antigenicity, even at low temperature (Table A2).

At high temperature, α_{S1}-CN antigenicity reduction was much greater, which could again be related to the extent of hydrolysis. Furthermore, it is clearly evident from the SDS PAGE gels (Fig. 2, Table 1) that αs_1-CN was very susceptible to hydrolysis at this temperature. The similar trend was reported previously when rise in temperature resulted in a greater degree of hydrolysis and greater reduction of antigenicity of milk proteins by latex peptidase (Oliveira et al., 2019).

Results of WPI hydrolysis at 60 °C also concur with the levels of β-Lg antigenicity, where the degree of change in antigenicity of β-LG was significantly greater (~46% antigenicity reduction) as compared to that whey protein hydrolysates obtained at 10 °C (Table 2). This could be related to the extent of hydrolysis as WPI hydrolysis at 60 °C showed highest %DH among all samples. In addition, shifting of the peaks in the FTIR interferograms has been observed (Table A1) indicating changes in the secondary structure that may have disrupted conformational epitopes and led to a slight reduction in antigenicity at low temperature. Due to very low increase in %DH, these changes were potentially more due to impact of temperature and to a lesser extent enzymatic action.

5. Conclusion

Actinidin was able to substantially reduce the antigenicity of αs_1-CN and β-Lg in a time and temperature dependent manner. Both substrates underwent greater hydrolysis at 60 °C than that at 10 °C with a strong negative correlation between %DH and antigenicity. Greater %DH resulted in greater reduction of antigenicity, likely due to cleavage of conformational epitopes. While actinidin was effective in hydrolysing αs_1-CN at both temperatures, this protease was not as effective during hydrolysis of β-Lg at low temperature. On the other hand, greatest %DH of β-Lg was achieved at 60 °C leading to significant antigenicity reduction. These results indicate that milk protein hydrolysates obtained by actinidin had reduced levels of antigenicity due to modifications of protein conformation or cleavage and masking of conformational and linear epitopes of the tested antigens. It could potentially be used in applications where reduction of allergenicity is required.

CRediT authorship contribution statement

S. Kaur: Conceived the study, Research question, Designed, Wrote the, Writing – original draft, Conceptualization, Writing – review & editing, Designed the tables, The figures, Methodology, Formal analysis, Investigation. **T. Huppertz:** Provided critical feedback, Formal analysis, Secured, Funding acquisition, Writing – review & editing, Supervised the study. **T. Vasiljevic:** Provided critical feedback, Formal analysis, Secured, Funding acquisition, Writing – review & editing, Supervised the study.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2022.113294.

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4.2 Supplementary material

Table A1. Proportion of defined structural elements of milk proteins observed within broad Amide I region (1700–1600 cm^{-1}) measured by FTIR after incubation of 5% MPC and WPI solutions with actinidin at a 1:100 enzyme to substrate ratio for up to 31 hours at 10 °C and up to 5 hours at 60 °C

Sample	Band assignment	Control	Time of hydrolysis (h)					
			0.16	2.5	5	10	22	31
		Peak area	Peak area	Peak area	Peak area	Peak area	Peak area	Peak area
		(%)	(%)	(%)	(%)	(%)	(%)	(%)
MPC/10 °C	β-sheet	44.5^{cC}	50.3^{aA}	42.3^{eA}	47.7^{bA}	43.3^{dA}	43.5^{dA}	42.8^{eA}
	Random coil	7.7^{dL}	10.8^{aL}	7.1^{eK}	7.1^{eJ}	10.2^{bF}	8.6^{cF}	7.7^{dE}
	α-helix	16.6^{fG}	17.7^{eG}	20.3^{bE}	20.2^{bE}	21.5^{aB}	19.8^{cC}	18.7^{dB}
	β-turn	15.7^{fH}	12.3^{gK}	26.0^{aC}	18.6^{dF}	19^{cC}	21.2^{bB}	16.4^{eC}
WPI/10 °C	β-sheet	50.7^{aA}	49.0^{bB}	42.6^{fA}	44.4^{cB}	43.8^{dA}	43^{eA}	42.1^{gB}
	Random coil	7.3^{bL}	5.7^{cO}	8.6^{aJ}	7.5^{bJ}	4.6^{dG}	8^{aG}	8.3^{aD}
	α-helix	17.2^{cF}	15.2^{eI}	18.9^{aF}	18.2^{bF}	16.6^{dD}	18.4^{bD}	18.8^{aB}
	β-turn	15.8^{cH}	14.7^{dJ}	16.1^{bG}	17.0^{aG}	14.3^{dE}	15.9^{cE}	16.5^{bC}
MPC/60 °C	β-sheet	44.4^{aC}	41.2^{bD}	41.5^{bB}	41.5^{bC}			
	Random coil	10.4^{aJ}	9.3^{bM}	10.0^{aI}	10.0^{aI}			
	α-helix	20.5^{bE}	20.1^{bE}	18.6^{cF}	21.3^{aD}			
	β-turn	12.8^{cI}	14.9^{bJ}	15.9^{aG}	15.0^{bH}			
WPI/60 °C	β-sheet	45.2^{aB}	42.1^{bC}	42.5^{bA}	41.9^{cC}			
	Random coil	9.3^{bK}	8.5^{cN}	10.0^{aI}	10.0^{aI}			
	α-helix	24.4^{aD}	18.6^{cF}	21.3^{bD}	21.0^{bD}			
	β-turn	13.0^{cI}	15.9^{aH}	14.7^{bH}	14.5^{bH}			

The pooled standard error of the mean of at least 3 independent observations was 0.158; lower case superscript letters indicate significant difference ($P < 0.05$) within a row and upper-case letters indicate significant difference ($P < 0.05$) within a column.

Table A2. Correlation coefficients describing the relationship between remaining antigenicity and residual intact β-LG or αs1-CN fraction. The quantity of individual proteins was estimated using the SDS-PAGE gels.

Sample/T	Remaining antigenicity/ Residual protein	
	β-LG	αs_1-CN
MPC/10 °C	0.960	0.896
MPC/60 °C	0.987	0.955
WPI/10 °C	0.957	-
WPI/60 °C	1.000	-

Chapter 5

Influence of Actinidin-Induced Hydrolysis on the Functional Properties of Milk Protein and Whey Protein Concentrates

Citation: Kaur, S., Vasiljevic, T., & Huppertz, T. (2023), *Foods,* 12, 3806. DOI: https://doi.org/10.3390/foods12203806

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DECLARATION OF CO-AUTHORSHIP AND CO-CONTRIBUTION: PAPERS INCORPORATED IN THESIS

This declaration is to be completed for each conjointly authored publication and placed at the beginning of the thesis chapter in which the publication appears.

1. PUBLICATION DETAILS (to be completed by the candidate)

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2. CANDIDATE DECLARATION

I declare that the publication above meets the requirements to be included in the thesis as outlined in the HDR Policy and related Procedures – policy.vu.edu.au.

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3. There are no other authors of the publication according to these criteria;

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Name(s) of Co-Author(s)	Contribution (%)	Nature of Contribution	Signature	Date
Surjit Kaur	80 %	Designing and conducting the experiment, data collection, statistical analysis and manuscript preparation	[redacted]	7/2/2024
Prof Todor Vasiljevic	10 %	Desinning the experiment, statistical analysis and manuscript editing		07/02/2024
Prof Thom Huppertz	10 %	Designing the experiment, manuscript editing and submission to the Journal		7/2/2024

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Influence of Actinidin-Induced Hydrolysis on the Functional Properties of Milk Protein and Whey Protein Concentrates

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Abstract: The main aim of the study was to establish the impact of limited proteolysis by actinidin on the functionality of selected milk protein systems. The plant protease actinidin was used to produce hydrolysates (MPHs) from milk protein concentrate (MPC) and whey protein concentrate (WPC) to 0, 5, 10 or 15% of the degree of hydrolysis (DH) at an enzyme-to-substrate ratio of 1:100 (5.21 units of actinidin activity g^{-1} of protein). The functionalities assessed included solubility, heat stability, emulsification and foaming properties. In general, significant changes in the functionalities of MPH were associated with the extent of hydrolysis. Solubility of hydrolysates increased with increasing %DH, with WPC showing about 97% solubility at 15% DH. Emulsifying properties were negatively affected by hydrolysis, whereas heat stability was improved in the case of WPC (~25% of heat stability increased with an increase in DH to 15%). Hydrolysates from both WPC and MPC had improved foaming properties in comparison to unhydrolysed controls. These results were also supported by changes in the FTIR spectra. Further adjustment of hydrolysis parameters, processing conditions and pH control could be a promising approach to manipulate selected functionalities of MPHs obtained using actinidin.

Keywords: actinidin; proteolysis; milk protein concentrate; whey protein concentrate; functional properties

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1. Introduction

Milk protein ingredients, including milk protein concentrate (MPC) and whey protein concentrate (WPC), are frequently used in nutritional and cultured dairy products and for protein standardisation and production of processed cheeses [1–3]. However, some of their applications in food systems are hindered by functionality issues. For example, high viscosity or poor solubility (at room temperature and neutral pH) leads to limitations with the utilisation of these proteins in high-energy drinks [3,4]. Furthermore, the emulsification and foaming properties of MPC are poorer than those of whey proteins (WPs), which can limit its usage in processed meats, soups, coffee creamers and whipped toppings [4,5]. Prolonged storage and elevated storage temperatures of milk protein powders such as MPC85 (containing 85% of proteins on dry matter) may lead to a rise in insolubility due to protein-protein interactions as a result of the creation of junction zones among adjacent protein powder particles [6]. Heat-induced destabilisation, especially of whey proteins, may cause phase separation or protein precipitation in the final products, such as in heat-treated beverage drinks [7].

Modifying functional properties, such as solubility, viscosity, emulsification and foaming, by enzymatic hydrolysis is one of the approaches to improve some of the properties of these proteins [8]. A study conducted by Ryan et al. [8] showed that protein hydrolysis rate resulted in greater solubility and reduced viscosity of milk protein isolate (MPI), which was

attributed to a change in the protein structure, size and hydrophobicity of the released peptides. Damodaran [9] suggested that many factors affect the foaming properties of proteins, such as the type of enzyme used, temperature, protein conformation and concentration, pH, mixing time, speed of whipping and foaming method. Furthermore, Banach et al. [2] also showed an improvement in nitrogen solubility of MPC80 hydrolysates after trypsin, pepsin, chymotrypsin or papain hydrolysis.

Current knowledge shows that changes in the functionality of milk protein hydrolysates depend on proteases used in their creation. While enzymes of animal and microbial origins have been used in the production and hydrolysis of dairy products, for example, chymosin, more attention has been placed more recently on plant-based proteases due to their availability and feasibility of extraction, especially from plant waste streams such as peels and rejects. Actinidin (EC 3.4.22.14), a plant-based cysteine protease (CA1) with a molecular weight of 23.5 kDa, is extracted from kiwi fruit. Actinidin can act in a wide range of temperatures (15–30 °C) and pH (4–10) with a broad substrate specificity [10]. The enzyme was recently assessed for potency to alleviate the antigenicity of two proteins in MPC and WPC, β-lactoglobulin and α_{s1}-casein [11]. The extent of antigenicity reduction was clearly dependent on the degree of hydrolysis. Furthermore, milk proteins appeared to be only partially hydrolysed by actinidin [10]. This limited hydrolysis clearly changes the conformation of the proteins in these preparations, which consequently indicates that their functionality may be affected as well [10,12,13]. For example, research has been conducted on the hydrolysis of milk protein concentrates with papain, an enzyme with a similar specificity to actinidin, which resulted in improved solubility at pH 7 [2]. Also, Al-Shamsi, Mudgil, Hassan, & Maqsood [14] showed substantially improved emulsification expressed as emulsifying activity index (EAI) when camel milk proteins were hydrolysed with papain as compared to that of the control. On the other hand, bromelain, a protease from pineapple, had no impact on EAI when used on the same substrate.

Therefore, the present study was carried out to establish whether milk protein hydrolysis by actinidin would have an impact on selected functional properties of MPC and WPC. The focus was on solubility, heat stability, foaming and emulsification properties, especially since the latter two are also related to solubility [15].

2. Materials and Methods

2.1. The Materials

WPC (80%, *w*/*w*, protein on dry matter) and MPC (80%, *w*/*w*, protein on dry matter) were obtained from Fonterra Cooperative (Palmerston North, New Zealand). Actinidin (KEP500 with 521 activity units g^{-1}) was kindly provided by kiwiEnzyme.com Ltd. (Martinborough, New Zealand). Trinitrobenzenesulfonic acid (TNBS), sodium phosphate buffer (0.2125 M, pH 8.2) and sodium dodecyl sulphate (SDS) were of analytical grade and were obtained from Sigma-Aldrich Pvt Ltd. (Castle Hill, NSW, Australia) and Merck KGa (Darmstadt, Germany). Simulated milk ultrafiltrate (SMUF, pH 7) [16] was used as a buffer during hydrolysis [17].

2.2. Sample Preparation and Enzymatic Hydrolysis of Milk Protein Systems

Protein dispersions (5%, *w*/*w*) were prepared by dispersing WPC or MPC in SMUF as described previously [10]. The control samples (0% DH) were prepared at room temperature (20 °C) in SMUF without the addition of the enzyme. Actinidin was added at the enzyme to substrate ratio (E:S) of 1:100 (5.21 units of actinidin activity per g of protein), and subsequently, each trial was performed at 60 °C until the 5, 10 or 15% degrees of hydrolysis (DH) was achieved. The %DH was assessed by the trinitrobenzenesulfonic acid (TNBS) procedure, as described previously [10]. The experimental design applied in the current study is depicted in Figure 1. Total protein was determined using a Kjeldahl method with a nitrogen conversion factor of 6.38 [18]. For functional properties, the controls were prepared under the same conditions (at 50 °C) but without enzymatic treatment. Hydrolysates were heat treated at 85 °C for 10 min without adding SDS to inactivate the enzyme [19,20]. The

samples were then freeze-dried using a pilot-scale freeze dryer (model FD-300, Airvac Engineering Pty. Ltd., Dandenong, Australia), followed by storing them in plastic airtight containers at ambient temperature for further analysis. For SDS-PAGE analysis, exactly 50 µL of the sample was preserved in 950 µL of SDS sample buffer and then stored at −20 °C for further testing.

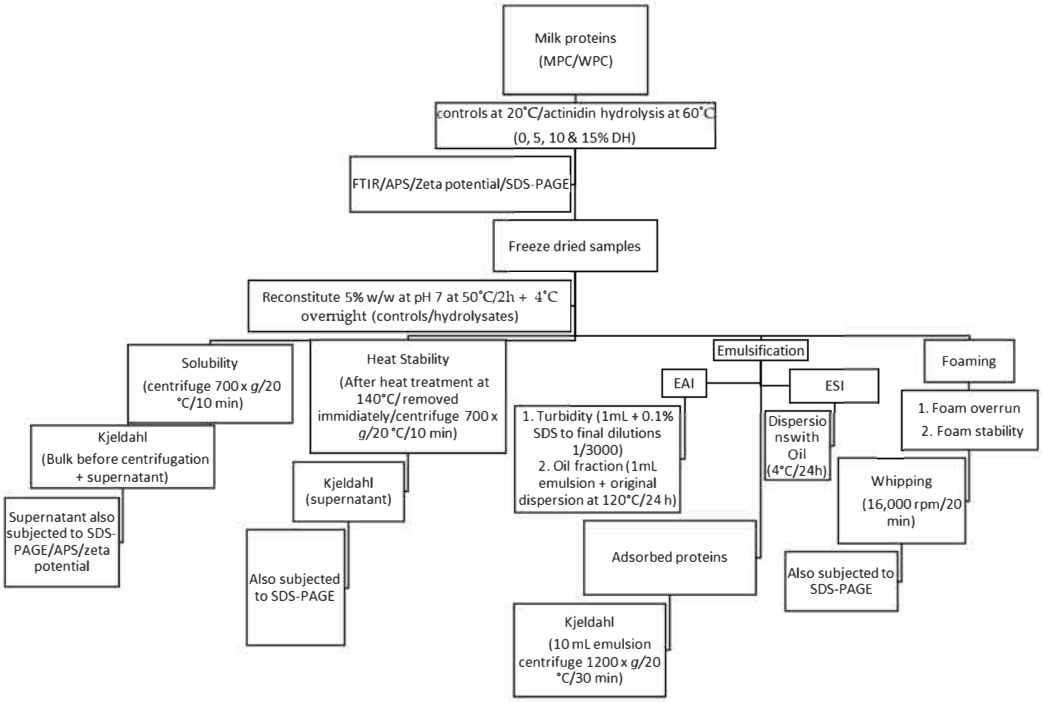

Figure 1. Experimental design used in the study. MPC = of milk protein concentrate; WPC = whey protein concentrate; FTIR = Fourier transform infrared spectroscopy; SDS-PAGE = Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis; APS = Average particle size; EAI = Emulsifying activity index; ESI = emulsion stability index.

2.3. Particle Size and Zeta Potential Measurement

Straight after hydrolysis, average particle size (APS) and zeta potential (ζ-potential) of all the controls and hydrolysed samples were determined by a Zetasizer-Nano ZS (Malvern Instruments, Malvern, UK) [21].

2.4. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The obtained hydrolysates were analysed by SDS-PAGE to study individual milk protein during hydrolysis. The analysis was performed under non-reducing and reducing (using β-mercaptoethanol) conditions as described previously [11]. Gels were scanned with the ChemiDoc imager (Chemidoc MP, Bio-Rad Laboratories, Hercules, CA, USA), and gels quantifications were performed for all reducing gels of both substrates in triplicate with software Image Lab 6.0.1 @2017, Bio-Rad Laboratories Inc.

2.5. Fourier Transform Infrared Spectroscopy (FTIR)

Immediately after treatment, FTIR spectra were obtained using a PerkinElmer Frontier FTIR spectrometer (PerkinElmer, Boston, MA, USA), as stated in our previous work [10]. Following peak areas were identified with four peak areas closely examined, including side chains (1607–1602 cm^{-1}), β-sheets (1640–1608 cm^{-1} and 1693–1680 cm^{-1}), random coils (1648–1642 cm^{-1}), α-helices (1663–1649 cm^{-1}), and β-turns (1678–1666 cm^{-1}) [22].

2.6. Functional Properties of MPs

The functional properties of freeze-dried powders were analysed by preparing 5% w/w (protein base) of the protein dispersions (controls and hydrolysed samples) at 50 °C for approximately 2 h under constant stirring, followed by overnight storage at 4 °C to allow for full hydration. The final weight was corrected with a pH adjustment to 7 using 1 M NaOH or 0.1 M HCl.

2.6.1. Determination of Protein Solubility

Each dispersion after hydrolysis of resuspended material after freeze-drying was centrifuged (Model J2HS; Beckman, Fullerton, CA, USA) at 700× g for 10 min at 20 °C [1], and their supernatants were collected. The protein content of their original dispersions and the resultant supernatants were then quantified by the Kjeldahl method using 6.38 as a conversion factor for both WPC and MPC [18], and protein solubility was expressed using the equation below [23].

These supernatants were also analysed by SDS-PAGE, and their APS and zeta potential were measured as described above.

$$\% \text{ Solubility } = \frac{\text{protein content ofsupernatant } (\text{mg mL}^{-1})}{\text{protein content of corresponding dispersion } (\text{mg mL}^{-1})} \cdot 100 \quad (1)$$

2.6.2. Determination of Heat Stability

The heat stability of protein dispersions was examined by establishing the solubility of the dispersions after exposure to a high temperature. A protocol described by Dissanayake et al. [23] was followed with samples treated in an oil bath at 140 °C. The time once samples reached 140 °C was recorded (WPC for 2.1 min; MPC for 2.66 min), and samples were immediately removed from a Riotek oil bath, followed by instant cooling in an ice slurry and centrifuged at 700× g for 10 min at 20 °C (Model J2HS). The protein quantification of supernatants of heated and original samples was conducted by the Kjeldahl method as per Section 2.6.1 using 6.38 as a conversion factor for both WPC and MPC (method 968.06) [18]. Heat stability was expressed using the equation below [23].

$$\% \text{ Heat stability } = \frac{\text{protein content in supernatant after heating } \left(\text{mg mL}^{-1}\right)}{\text{protein content of correspondingsupernatant prior to heating } (\text{mg mL}^{-1})} \cdot 100 \quad (2)$$

2.6.3. Determination of Emulsifying Properties

Emulsifying activity index (EAI), emulsion stability index (ESI) and protein adsorption of each sample were analysed by a turbidimetric technique described by Cameron, Weber, Idziak, Neufeld, & Cooper [24] and modified by Dissanayake et al. [23]. The EAI of samples were calculated using the following equation expressed as units of area of interface stabilised per unit weight of protein:

$$\text{EAI} = \frac{2 \cdot \text{T}}{(1 - \Phi) \cdot \text{C}} \quad (3)$$

where T denotes turbidity, Φ is the oil volume fraction, and C is the weight of protein per unit volume of aqueous phase before an emulsion is formed.

ESI was estimated after holding the emulsions at 4 °C for 24 h using the following formula:

$$\text{ESI} = \frac{(\text{T} \cdot \Delta\text{t})}{\Delta\text{T}} \quad (4)$$

where T is the turbidity value at zero h; Δt is the time interval in hrs; ΔT is turbidity after Δt [25].

The amount of adsorbed protein was calculated by the equation:

$$\begin{aligned}\text{Adsorbed protein } \left(\text{mg mL}^{-1}\right) &= \text{protein in stock solution } \left(\text{mg mL}^{-1}\right) \\ &- \text{protein in aqueous layer of emulsion } \left(\text{mg mL}^{-1}\right)\end{aligned} \quad (5)$$

2.6.4. Determination of Foaming Properties

Foaming properties were determined according to the method described by Phillips et al. [26] with minor modifications stated by Dissanayake et al. [23]. Foam overrun was calculated using the following equation:

$$\text{Overrun } (\%) = \frac{(\text{wt. of 100 mL sample suspension}) - (\text{wt. of 100 mL foam})}{\text{wt. of 100 mL foam}} \cdot 100 \quad (6)$$

Foam stability was measured by monitoring the drainage of liquid at ambient temperature, as described by Dissanayake et al. [23]. It was defined as a time to attain 50% drainage of the original weight of the dispersion [26].

2.7. Statistical Analysis

The experiments were conducted in a randomised split block design with the extent of hydrolysis as the main factor and repetitions as the block. The design was replicated at least three times on separate occasions for both substrates, and the data were expressed as the mean ± SD of three independent assays. In addition, for the Kjeldahl analysis, the analytical determination was replicated twice, followed by a subsampling (n = 4). The data was analysed by two-way ANOVA using the SAS software (v. 9.1). The means were compared using the Tukey multi-comparison, and the significance level was set at $p < 0.05$.

3. Results

3.1. Changes in Particle Size and Composition of Milk Protein Hydrolysates Obtained by Actinidin-Induced Hydrolysis of Milk Protein Concentrate and Whey Protein Concentrate

As Table 1 indicates, the bulk of the MPC control had an average particle size of 295 nm, which rose with an increase in %DH, reaching 343 nm at 15%DH. At the same time, the average size of the particles in the MPC supernatant was reduced significantly from that of the control (282 nm) down to 171 nm. On the contrary, the average particle size of both bulk and supernatant of the WPC control was 426 and 386 nm and declined to 410 and 353 nm, respectively, with an increase in DH (15%). However, this particle size (as large as fat globules) can be attributed to fat globules size due to the presence of residual lipid content in WPC powder [27].

Also, as indicated by Table 1, the zeta potential of both bulk and supernatants of WPC control was −9 mV. After attaining 15% DH, zeta potential became more negative, reaching −13 and −14 mV for the bulk and supernatants, respectively. Similarly, the MPC underwent a comparable increase in negative zeta potential from −15 mV (0% DH) to −18 mV (15% DH) for the supernatants. However, the MPC bulk had no apparent trend, which could be assigned to the heterogeneity of proteins as opposed to that of WPC; thus, the changes may have been various.

The PAGE patterns of MPC and WPC hydrolysates are shown in Figures 2–4, which demonstrate the nature and extent of protein interactions. Protein patterns were compared, and they appear to be in agreement with the %DH and solubility (Figures 2–4; Tables 2–4). As expected, casein bands (α_S-, β-, and κ-CN) were detected in the MPC samples only, and those of major whey proteins, β-LG and α-LA, were detected in both MPC and WPC samples. At the highest DH (15%), there was <10% and 5% of each casein remaining after hydrolysis in the case of MPC bulk and supernatant, respectively (Figure 2B; Table 2). In the WPC bulk (Figure 4D; Table 2), ~21% and ~25% of β-LG and α-LA remained, whereas

no bands were detected in the WPC supernatants, indicating that released peptides were not retained in the gel.

Table 1. Average particle diameter and zeta potential of the whole samples and supernatants of milk protein hydrolysates (MPH) obtained from milk protein concentrate (MPC) and whey protein concentrate (WPC) incubated with actinidin to a degree of hydrolysis (%DH) of 0, 5, 10 or 15 at 60 °C.

MPH (%DH)	Particle Diameter (nm)				Zeta Potential (mV)			
	MPC		WPC		MPC		WPC	
	MPC Bulk	MPC Supernatant	WPC Bulk	WPC Supernatant	MPC Bulk	MPC Supernatant	WPC Bulk	WPC Supernatant
0	295 ± 4^{b}	282 ± 4^{a}	426 ± 1^{a}	386 ± 3^{a}	-2 ± 0.3^{a}	-15 ± 0.0^{ab}	-9 ± 0.0^{a}	-9 ± 0.4^{a}
5	281 ± 2^{d}	233 ± 12^{b}	419 ± 3^{b}	381 ± 1^{b}	-4 ± 0.6^{b}	-16 ± 1.3^{b}	-10 ± 0.4^{b}	-10 ± 0.4^{b}
10	290 ± 3^{c}	180 ± 8^{c}	409 ± 4^{c}	365 ± 1^{c}	-7 ± 0.0^{c}	-14 ± 0.2^{a}	-10 ± 1.9^{b}	-12 ± 2.2^{c}
15	343 ± 5^{a}	171 ± 5^{d}	410 ± 3^{c}	353 ± 3^{d}	-3 ± 1.9^{ab}	-18 ± 0.0^{c}	-13 ± 0.4^{c}	-14 ± 0.2^{d}

The values are presented as means of subsampling of three independent observations plus or minus standard deviation (SD). The values with different lower-case letters indicate significant differences ($p < 0.05$) within a column.

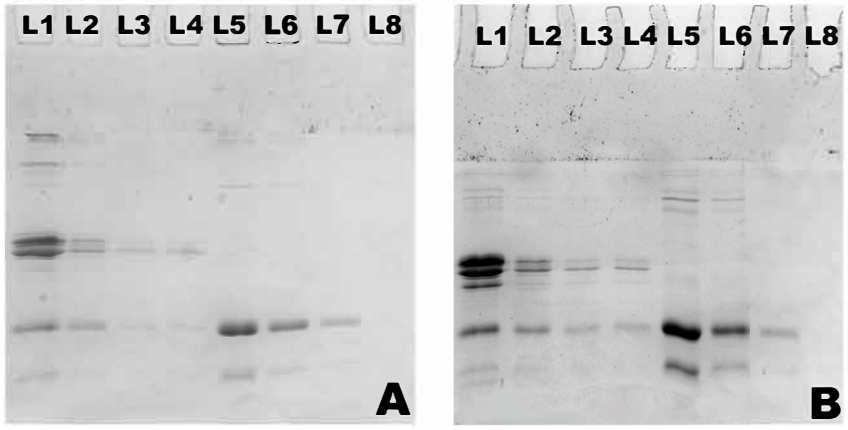

Figure 2. Non-reducing (**A**) and reducing (**B**) SDS-PAGE patterns of hydrolysates of heated supernatants for heat stability of MPC (L1–L4) and WPC (L5–L8) obtained by actinidin treatments at 60 °C with 0 (L1, L5), 5 (L2, L6), 10 (L3, L7) and 15% DH (L4, L8).

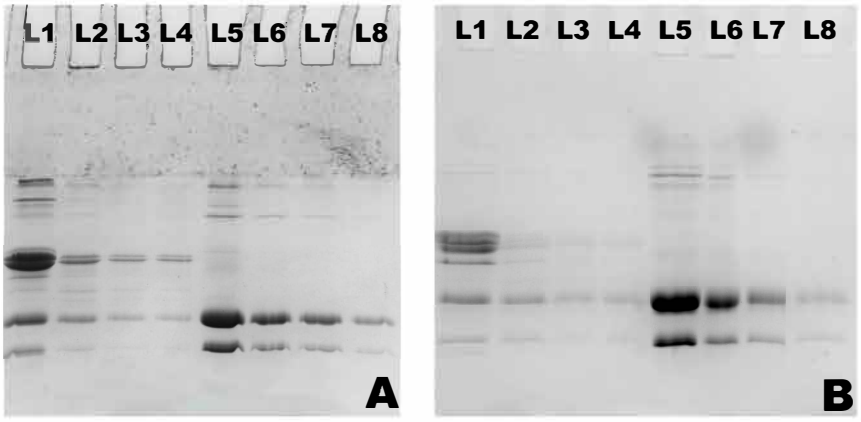

Figure 3. Non-reducing (**A**) and reducing (**B**) SDS-PAGE patterns of MPC (L1–L4) and WPC (L5–L8) hydrolysates of drained liquid for foaming obtained by actinidin treatments at 60 °C with 0 (L1, L5), 5 (L2, L6), 10 (L3, L7) and 15% DH (L4, L8).

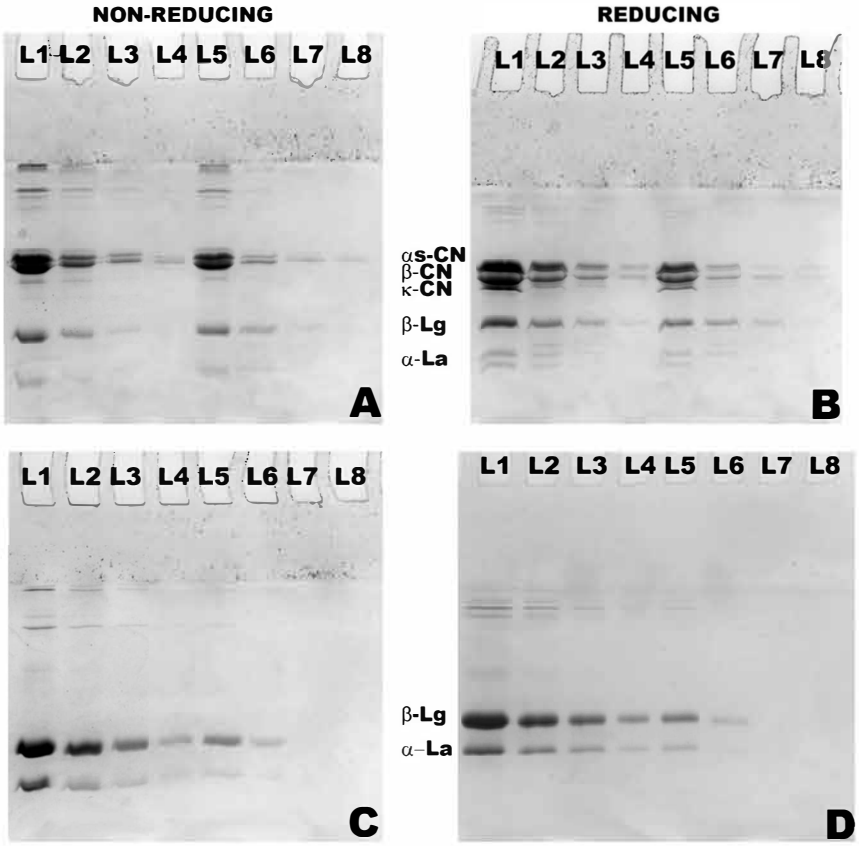

Figure 4. Non-reducing (**A**,**C**) and reducing (**B**,**D**) SDS-PAGE patterns of hydrolysates of original (L1–L4) and supernatants (L5–L8) for solubility of MPC (**A**,**B**) and WPC (**C**,**D**) obtained by actinidin treatments at 60 °C with 0 (L1, L5), 5 (L2, L6), 10 (L3, L7) and 15% DH (L4, L8).

Table 2. Residual intact milk proteins in the whole samples and supernatant of dispersions of milk protein concentrate (MPC) and whey protein concentrate (WPC) incubated with actinidin to a degree of hydrolysis (%DH) of 0, 5, 10 and 15 at 60 °C.

Protein	Proportion of Proteins Remaining Relative to Control (%)					
	DH (%)					
	5	10	15	5	10	15
MPC	Solubility					
	Whole Sample			Supernatant		
α_s-CN	54.6 ± 3.2 ^{aE}	27.4 ± 2.1 ^{cD}	8.1 ± 0.5 ^{eD}	38.7 ± 3.4 ^{bC}	14.8 ± 0.9 ^{dB}	3.0 ± 0.2 ^{fB}
β-CN	57.4 ± 0.0 ^{aD}	30.5 ± 0.0 ^{bC}	7.0 ± 0.0 ^{eE}	27.0 ± 0.0 ^{cE}	19.2 ± 0.0 ^{dA}	4.6 ± 3.6 ^{fA}
κ-CN	43.7 ± 0.1 ^{aG}	19.8 ± 0.0 ^{bE}	5.9 ± 0.0 ^{dF}	20.2 ± 0.0 ^{bG}	7.0 ± 0.0 ^{cD}	0.0 ± 0.0 ^{eD}
β-LG	61.5 ± 3.6 ^{bC}	31.9 ± 3.3 ^{cC}	9.6 ± 1.0 ^{eC}	63.7 ± 3.5 ^{aA}	13.4 ± 0.0 ^{dC}	2.0 ± 0.0 ^{fC}
α-LA	46.0 ± 4.1 ^{aF}	18.3 ± 1.7 ^{cF}	0.0 ± 0.0 ^{dG}	43.0 ± 1.3 ^{bB}	0.0 ± 0.0 ^{dE}	0.0 ± 0.0 ^{dD}
WPC	Whole sample			Supernatant		
β-LG	81.2 ± 3.9 ^{aB}	60.6 ± 1.7 ^{bA}	25.5 ± 0.5 ^{dA}	36.8 ± 0.5 ^{cD}	19.0 ± 0.0 ^{eA}	0.0 ± 0.0 ^{fD}
α-LA	83.5 ± 1.6 ^{aA}	45.7 ± 2.0 ^{bB}	21.5 ± 0.5 ^{dB}	23.9 ± 0.6 ^{cF}	0.0 ± 0.0 ^{eE}	0.0 ± 0.0 ^{eD}

The values are presented as means of at least three independent observations ± standard deviation (SD); lower and upper-case superscript letters indicate significant differences ($p < 0.05$) within a row and a column, respectively.

Table 3. Proportion (%) of milk proteins remaining (relative to control) after heat stability of varying degrees of hydrolysis of MPC or WPC by actinidin at 60 °C. Where (-) is not determined.

Proportion of Proteins Remaining Relative to Control (%) during Heat Stability						
DH (%)	**5**	**10**	**15**	**5**	**10**	**15**
Protein	**Supernatant (MPC)**			**Supernatant (WPC)**		
α_s-CN	28.2 ± 2.1^{aD}	13.4 ± 0.4^{bC}	10.8 ± 0.3^{cC}	-	-	-
β-CN	32.3 ± 1.8^{aC}	21.0 ± 1.6^{bB}	20.0 ± 1.7^{cB}	-	-	-
κ-CN	28.0 ± 1.3^{aD}	11.1 ± 0.9^{bD}	9.7 ± 0.9^{cD}	-	-	-
β-LG	51.7 ± 2.6^{aA}	32.3 ± 2.2^{cA}	25.7 ± 2.3^{dA}	48.5 ± 3.1^{bA}	22.3 ± 2.5^{eA}	0.0 ± 0.0^{fA}
α-LA	38.0 ± 2.8^{bB}	0.0 ± 0.0^{dE}	0.0 ± 0.0^{dE}	47.7 ± 2.1^{aB}	11.3 ± 4.5^{cB}	0.0 ± 0.0^{dA}

The values are means of at least three independent observations ± standard deviation (SD); lower and upper-case superscript letters indicate significant differences ($p < 0.05$) within a row and a column, respectively.

Table 4. Proportion (%) of milk proteins remaining (relative to unhydrolysed control) after foaming. Where (-) is not applicable.

Proportion of Proteins Remaining Relative to Control (%) during Foaming						
DH (%)	**5**	**10**	**15**	**5**	**10**	**15**
Protein	**Foam Drained Liquid (MPC)**			**Foam Drained Liquid (WPC)**		
α_s-CN	18.0 ± 1.3^{aE}	7.8 ± 0.3^{bD}	3.4 ± 0.2^{cD}	-	-	-
β-CN	27.1 ± 0.5^{aC}	13.8 ± 0.5^{bB}	2.8 ± 0.0^{cE}	-	-	-
κ-CN	26.3 ± 1.2^{aD}	11.3 ± 0.0^{bC}	5.0 ± 0.3^{cC}	-	-	-
β-LG	33.2 ± 2.4^{aB}	15.1 ± 0.0^{cA}	9.4 ± 0.0^{dA}	33.8 ± 0.7^{aA}	18.7 ± 1.5^{bB}	15.4 ± 0.6^{cA}
α-LA	36.8 ± 0.3^{aA}	11.6 ± 0.0^{dC}	7.3 ± 0.0^{eB}	26.4 ± 2.4^{bB}	25.7 ± 2.3^{cA}	4.7 ± 0.4^{fB}

Values are means of at least three independent observations ± standard deviation (SD); upper-case and lower-case superscript letters indicate significant differences ($p < 0.05$) within a column and a row, respectively.

3.2. Modification of Secondary Structure in Milk Protein Hydrolysates Obtained by Actinidin

All peaks assigned to a specific FTIR region were selected carefully, including the otherwise hidden peaks that were only possible to see in spectra in a stacking form of peaks (Supplementary Materials) to determine the main proteins' structural changes during processing and their interactions. The current study showed an inverse trend in the peak areas for β-sheets and α-helical structures, while negligible changes for random coils and β-turns took place for both MPC and WPC (Table 5). In the case of the MPC, the peak areas assigned to β-sheets significantly increased ($p < 0.05$), approximately by about 6% and 11% in the samples with 10% and 15% DH, respectively, in comparison to that of the control. Simultaneously, the sample with 10% DH demonstrated a substantial reduction of α-helix peak area by ~2%, with a further ~6% decrease in this peak area when the sample was further hydrolysed (15% DH), compared to that of the control. In the case of the WPC, a rise of ~4% in the peak areas associated with β-sheets was observed in both 10–15% DH samples compared to that of the control. At the same time, the α-helix peak area decreased by ~5% at the maximum DH compared to the control. Interestingly, this study showed a clear trend, but contrary to previously reported FTIR data, where only limited changes in the secondary structure of MPC and WPI were observed [11].

Table 5. Proportion of defined structural elements of milk proteins observed within broad amide I region (1700–1600 cm^{-1}) measured by FTIR after hydrolysis (MPH) of milk protein concentrate (MPC) and whey protein concentrate (WPC) by actinidin to 0, 5, 10 and 15% DH at 60 °C.

MPH	Band Assignment	Degree of Hydrolysis (%)							
		Control (0)		5		10		15	
		Band Frequency (cm^{-2})	Peak Area %	Band Frequency (cm^{-2})	Peak Area %	Band Frequency (cm^{-2})	Peak Area %	Band Frequency (cm^{-2})	Peak Area %
MPC	β-sheet	1638–1608, 1691–1681	42.4 ± 8.4^{dB}	1640–1608, 1693–1681	45.2 ± 1.2^{cB}	1640–1609, 1693–1681	47.7 ± 3.1^{bB}	1640–1610, 1693–1682	53.2 ± 1.0^{aA}
	Random coil	1647–1643	10.6 ± 1.9^{bG}	1648–1643	11.6 ± 4.3^{aG}	1646–1642	8.4 ± 1.4^{cG}	1646–1642	8.2 ± 0.4^{cD}
	α-helix	1662–1651	22.1 ± 3.3^{aC}	1662–1650	22.5 ± 1.4^{aC}	1662–1650	19.8 ± 1.6^{bC}	1662–1650	15.9 ± 0.8^{cB}
	β-turn	1677–1666	17.4 ± 1.2^{bE}	1678–1667	17.6 ± 5.3^{bE}	1678–1667	18.8 ± 1.4^{aD}	1677–1666	16.1 ± 1.4^{cB}
WPC	β-sheet	1639–1610, 1691–1681	49.6 ± 1.3^{bA}	1639–1610, 1691–1680	49.6 ± 0.8^{bA}	1640–1610, 1690–1681	53.3 ± 5.0^{aA}	1640–1610, 1691–1681	53.4 ± 1.5^{aA}
	Random coil	1648–1643	9.0 ± 0.7^{bH}	1647–1643	11.2 ± 0.4^{aG}	1646–1642	8.7 ± 1.8^{bG}	1646–1642	9.0 ± 0.3^{bC}
	α-helix	1663–1650	20.6 ± 1.3^{aD}	1663–1650	18.3 ± 1.5^{bD}	1661–1649	14.8 ± 3.2^{dE}	1660–1649	15.9 ± 1.1^{cB}
	β-turn	1677–1666	15.5 ± 1.4^{bF}	1678–1667	16.8 ± 1.2^{aF}	1677–1666	11.7 ± 2.4^{cF}	1678–1666	15.8 ± 2.0^{bB}

Values are means of at least three independent observations ± standard deviation (SD); The lower-case superscript letters indicate significant differences ($p < 0.05$) within a row, and the upper-case letters indicate significant differences ($p < 0.05$) within a column.

3.3. Functional Properties of Hydrolysates

3.3.1. Functional Properties of MPC Hydrolysed by Actinidin

Enzymatic hydrolysis affected the solubility of MPC in a % DH-dependent manner. The solubility of the control sample was about 50%, which was improved to ~60% and the most to 65% at 5% and 15% DH, respectively (Figure 5). Heat stability appeared to follow the same pattern—greater solubility led to improved heat stability (Figure 6). However, heat stability was not clearly dependent on the %DH. The control was characterised with 90.7% heat stability, which further improved to 95.4% when MPC was treated to 5% DH. This was basically the maximum heat stability the hydrolysed samples were able to reach as other samples at greater %DH remained at this level (Figure 3 and Table 3). This can also be seen in Figure 3B and Table 3, in which MPC supernatant at 15%DH showed total disappearance of bands taking place only in the case of α-LA and about 10% of αs- and κ-CN remained along with about 20% of β-CN and 26% β-LG.

The emulsifying activity index (EAI) of the MPC samples significantly ($p < 0.05$) decreased with an increase in %DH. The control MPC sample was characterised with the greatest EAI (17.45 $m^2\ g^{-1}$), which decreased to 13.80 $m^2\ g^{-1}$, 11.58 $m^2\ g^{-1}$ and the lowest to 9.49 $m^2\ g^{-1}$ upon hydrolysis to 5%, 10% and 15% DH, respectively (Table 6). Furthermore, the emulsion stability of all samples appeared to be between ~22 to 24 h, with the control having the greatest ESI of 23.9 h and the lowest of 22.5 h was observed for samples with the highest %DH. Simultaneously, a significant drop was observed in the concentration of adsorbed protein on the surface of oil droplets as it declined from 2.33 $mg^{-1}\ mL^{-1}$ for the control to 1.50 $mg^{-1}\ mL^{-1}$ for the actinidin-treated sample with 15% DH (Table 6) indicating a poorer surface coverage that likely resulted in diminished EAI. On the other hand, foam overrun and foam stability improved with the hydrolysis rate. Foam overrun increased from approximately 344% for the control to 406% for the sample with 15% DH. The foam stability of the sample was also improved, increasing from 1260 s to 2454 s (Table 6). Also, the protein patterns of the foam-drained liquid of MPC obtained during the foaming analysis are shown in Figure 4B and Table 4. In the MPC drained liquid, ~3% of αs- and β-CN, 5% of κ-CN, 9% of β-LG and 7% of α-LA remained at 15%DH.

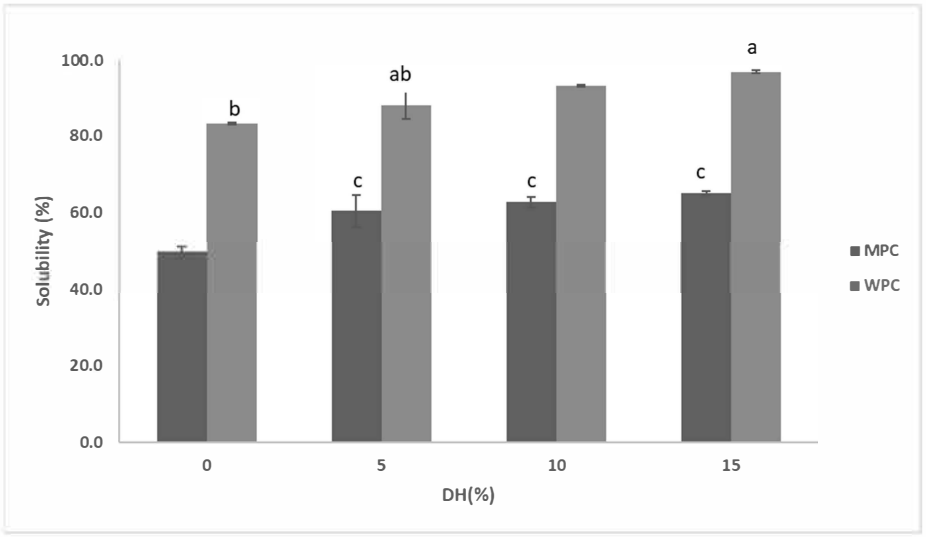

Figure 5. Solubility percentage of hydrolysates of 5% (*w*/*w*) dispersions of MPC and WPC with actinidin at 60 °C at 0, 5, 10 and 15%DH. The values with different lower-case letters indicate significant differences ($p < 0.05$).

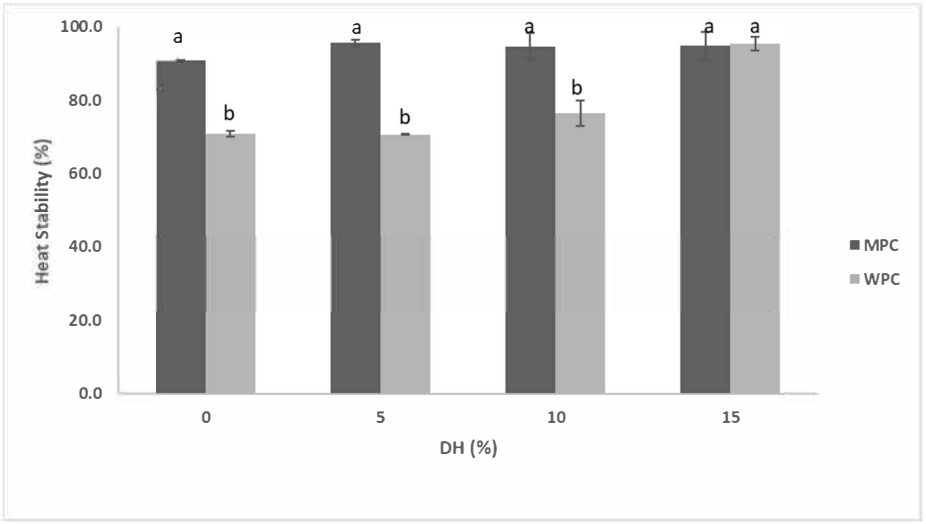

Figure 6. Heat stability % of hydrolysates of 5% (*w*/*w*) dispersions of MPC and WPC with actinidin at 60 °C at 0, 5, 10 and 15% DH. The values with different lower-case letters are significantly different ($p < 0.05$).

Table 6. Adsorbed proteins, emulsifying and foaming properties of milk protein hydrolysates (MPH) obtained from milk protein concentrate (MPC) and whey protein concentrate (WPC) by actinidin to 0, 5, 10 and 15% DH at 60 °C.

MPH (%DH)	Foam Stability (s)	Overrun (%)	*EAI* ($m^2\ g^{-1}$)	Adsorbed Protein ($mg^{-1}\ mL^{-1}$)	*ESI* (h)
Hydrolysates from MPC					
0	1260 ± 8^{d}	344.8 ± 4^{c}	17.45 ± 0.0^{c}	2.33 ± 0.2^{a}	23.9 ± 0.1^{d}
5	1904 ± 10^{c}	349.7 ± 3^{c}	13.80 ± 0.4^{d}	1.72 ± 0.1^{b}	23.8 ± 0.1^{d}
10	2160 ± 6^{b}	358.5 ± 2^{b}	11.58 ± 0.8^{e}	1.56 ± 0.1^{bc}	22.9 ± 0.2^{e}
15	2454 ± 2^{a}	406 ± 3^{a}	9.49 ± 0.1^{f}	1.50 ± 0.2^{c}	22.5 ± 0.5^{f}
Hydrolysates from WPC					
0	7 ± 1^{h}	0 ± 0.0^{f}	20.33 ± 0.3^{a}	0.59 ± 0.1^{f}	25.1 ± 0.5^{a}
5	120 ± 2^{e}	247.6 ± 4^{e}	20.24 ± 0.9^{a}	0.73 ± 0.1^{de}	24.8 ± 0.7^{b}
10	105 ± 6^{f}	252.1 ± 4^{e}	19.22 ± 0.7^{b}	0.77 ± 0.1^{d}	24.6 ± 0.4^{bc}
15	40 ± 4^{g}	270.4 ± 3^{d}	17.45 ± 0.2^{c}	0.80 ± 0.0^{d}	24.5 ± 0.3^{bc}

The values are the mean of at least three independent observations ± standard deviation (SD); lower-case superscript letters indicate significant differences ($p < 0.05$) within a column.

3.3.2. Functional Properties of WPC Hydrolysates Obtained by Actinidin

In the case of WPC (Figure 5), a similar trend to that of MPC was observed in relation to the solubility, which increased concomitantly with %DH. The untreated sample had about 83% solubility, which increased to about 88%, 93% and 97% at 5%, 10% and 15% DH, respectively (Figure 5). Proteolysis also significantly improved the heat stability, especially at its highest %DH. For example, from the control, heat stability increased from ~ 71% to the highest of ~95.1% at 15% DH (Figure 6). It can also be seen from Figure 3B and Table 3 that heat-treated hydrolysates had no visible bands after SDS-PAGE analysis of the WPC supernatant. However, at 0 and 5% DH, heat stability did not differ significantly ($p > 0.05$), and even at 10% DH, only about a 6% increase was observed, indicating that substantial hydrolysis was required to improve this functionality.

The EAI of the WPC control was 20.33 $m^2\ g^{-1}$ and decreased to 17.45 $m^2\ g^{-1}$ for the samples obtained after 15%DH. Also, there was a negligible change of EAI at 5% DH or only ~<1 of EAI change at 10% DH (Table 6). Furthermore, emulsion stability declined from 25.1 (control) to 24.5 h (hydrolysates with 15% DH). In contrast to MPC, WPC hydrolysates showed an increase in the concentration of adsorbed protein on the surface of oil droplets (Table 6) as the amount of proteins increased from 0.59 $mg^{-1}\ mL^{-1}$ (control) to 0.80 $mg^{-1}\ mL^{-1}$ (sample with highest DH).

Interestingly, the WPC control did not foam (overrun of 0%) under the experimental conditions, which was similar to the studies conducted by Dissanayake & Vasiljevic [28], reporting 0 s foam stability for whey proteins control sample, and Althouse, Dinakar, & Kilara, [29], where control whey protein isolate retentate showed no stable foam formation and 0% foam overrun. However, a great improvement in foaming of WPC was achieved to about 247, 252 and 270%, concomitant with an increase in %DH to 5, 10 and 15%, respectively. However, this increase in the foam overrun was accompanied by compromised foam stability. The most stable foam was the one with the lowest %DH (120 s), while the least stable foam was generated from the dispersion containing WPC with 15% DH (40 s). Similarly, the protein patterns of foam-drained liquid of WPC resulted in about 15% of β-LG and only about 5% of α-LA fractions remaining at 15% DH (Figure 4B and Table 4).

4. Discussion

Our previous studies showed that actinidin can be used to hydrolyse milk proteins to a certain extent, and the hydrolysates obtained had lower antigenicity in the case of

both WPC and MPC substrates [10,11]. However, the use of actinidin for modulating the functional properties of dairy systems has not been assessed and applied. Therefore, the present study was carried out to explore the effect of actinidin hydrolysis on functional properties of commercial MPC and WPC, including solubility, heat stability, foaming and emulsification, as the latter two properties are also related to solubility [15].

Milk proteins have broad applicability in various food products due to their nutritional or physical properties. However, their application in food systems may be limited due to several important issues. For example, whey proteins are very soluble, a property highly dependent on the pH and/or temperature of the system, which creates problems during downstream processing and especially during manufacturing involving heat applications. Thus, partial hydrolysis may improve the stability of whey proteins by increasing their solubility and, thus, heat stability [15]. Also, milk protein concentrate usually has poor solubility, especially after prolonged storage [6], which may limit its functional properties. Thus, hydrolysis of milk proteins by proteases has the potential to address some of the issues leading to compromised functionalities [8]. Several studies have applied controlled enzymatic hydrolysis to enhance the functional properties of whey proteins, caseins and milk protein isolates. It has been observed that with a greater degree of hydrolysis, solubility can be increased with a concomitant decrease in viscosity [2,8,30].

Native WPs are globular with higher numbers of surface hydrophilic amino acid residues and buried hydrophobic and cysteine groups, resulting in high aqueous solubility [31,32]. In the case of MPC, poor solubility mainly occurs due to the structural rearrangement of the casein micelles that have a high hydrophobicity index [33]. Hydrophobic interactions, which take place between hydrophobic regions of caseins, are the main drivers of MPC insolubility [1,6,34].

Hydrolysis improved the solubility of both MPC and WPC further with the elevation of %DH (Figure 5), with whey proteins achieving almost full solubility at 15% DH. Even in MPC dispersions, about a 15% rise in solubility was observed at 15% DH in comparison to that of the control. Furthermore, the improvement in solubility can also be compared to the zeta potential of MPH in the case of both substrates. Hydrolysates were characterised by a greater net-negative zeta potential compared to that of the controls, where both bulk and supernatant of both substrates mostly resulted in greater negative zeta potential and, thereby, higher solubility through likely enhanced electrostatic repulsions. The changes in solubility were also reflected in the electrophoretic patterns of both substrates. α_s-CN in the case of caseins and α-LA in the case of whey proteins were mostly affected fractions, which resulted in hydrolysates with smaller molecular weight oligopeptides with an increase in %DH, as observed in the SDS-PAGE gels, which consequently resulted in improved solubility. However, while hydrolysis improved solubility, which can also be related to reduced average particle size, the extent of proteolysis appears to be also relevant as the greater DH, i.e., 15% (MPC bulk), resulted in a substantial rise in the average particle size (up to 343 nm from 295 nm) likely indicating aggregation. However, this aggregation was not visible in the gels. This can be due to the nature of these aggregates, as they could have been created via weak forces easily broken by SDS. During proteolysis, cleavage of peptide bonds takes, which causes a release of the number of amino and carboxyl groups, resulting in an increment in hydrophilicity and net charge density of the hydrolysates obtained by promoting proteins-water interactions [35].

According to previous studies, β-sheet and α helix contents of native and unhydrolysed β-lactoglobulin comprise about 43–50% and 10–15% of all structural elements, respectively [36]. On the other hand, α-LA has about 18% and 36% [36], α_{s2}-CN about 27% and 32% [37], β-CN about 34% and 29% [37], κ-CN has about 39–41% and 8–10% of these structural elements [38], respectively. Furthermore, αs_1-CN has only a small amount of secondary structure containing only α-helices or β-sheets. In the current study, despite negligible change in the contents of β-turn and random coils, in the case of both substrates at maximum DH, a significant change was seen in the content of β-sheet (increased) and α-helical (declined) in comparison to the unhydrolysed samples. This implies that the

actinidin hydrolysis may have resulted in conformational rearrangements, transforming these proteins from a predominant α-helical to a β-sheet form. Both β-sheet and α-helical structures are mainly created by hydrogen bonds between amine hydrogen and carbonyl oxygen atoms that construct the peptide backbone of the substrates [36]. The FTIR analysis (Table 5) showed that the protein structure was changed substantially, which likely led to the exposure of previously buried hydrophilic regions to the environment [15] and consequently improved solubility. It can also be seen in Supplementary Materials Figure S1, where spectra of WPC hydrolysates containing 15% DH showed the highest peaks.

Furthermore, significant increases in heat stability for whey proteins after hydrolysis are in agreement with a study conducted by Castro & Sato [39], in which high solubility and high heat stability were recorded after hydrolysis of whey proteins with Flavourzyme®. For WPC, it is critical to have appropriate heat stability as these proteins usually go through reconstitution and heat treatment during manufacturing, which may cause end-product destabilisation. In the case of MPC, heat instability occurs regardless of the fact that caseins can withstand higher temperature treatment without aggregation. Heat instability is also caused by whey proteins (mostly driven by β-LG due to its higher concentration in whey) denaturation and their reaction with casein micelles [40]. κ-CN and β-LG complex (colloidal or serum) are associated with regions of maximum and minimum heat stability, respectively [41,42]. Various studies have been conducted on milk proteins with the main focus on their heat stability [1,3,43]. Also, a study conducted by Gauthier & Pouliot [44] showed improved heat stability of hydrolysed whey proteins in an acidic beverage.

While notable improvement in solubility and heat stability has been observed upon substrate hydrolysis using actinidin, both substrates experienced a reduction in EAI and ESI. A similar trend has been reported by Slattery & Fitzgerald [45] when sodium caseinate hydrolysates were obtained by *Bacillus* proteinase and another study by Singh & Dalgleish [46] when commercial range of whey protein hydrolysates was tested for emulsifying properties. Emulsifying properties mainly depend on both surface hydrophobicity and molecular flexibility of proteins [47]. The greater emulsifying activity index appears with improvement in interfacial properties due to partial unfolding of proteins [48]. A greater amount of adsorbed proteins was present in the case of whey proteins as DH increased. However, greater hydrolysis of MPC resulted in a lesser amount of adsorbed proteins on the surface of fat droplets, which led to poorer emulsification. Reduction in emulsification activity occurred either due to the presence of a greater amount of hydrophilic peptides that lack or limit binding to the oil–water interface or the absence of a strong interfacial layer to prevent recoalescence of the oil [15]. It is well known that α-LA has poor gelling but good emulsifying properties, whereas β-LG exhibits excellent gelling, foaming and emulsifying properties [25]. In the current study, the progressive disappearance of α-LA bands in Figures 2D and 3B demonstrate preferential hydrolysis of this protein fraction to smaller peptides, hence likely reduction of EAI and their stability. Also, emulsion stability was slightly decreased in the case of both substrates compared to their controls. Lower ESI may be due to a rise in the number of polar groups resulting from proteolysis, which altered a protein structure and thus enhanced hydrophilicity. A similar trend has been reported previously by Severin & Xia [15] and Singh & Dalgleish [46] when they used whey protein hydrolysates, and Slattery & Fitzgerald [45] used sodium caseinate hydrolysates created by different proteases.

According to Damodaran [9], partial hydrolysis of milk proteins generally improves foaming properties, whereas extensive hydrolysis can adversely affect it. In the current study, improvement in foam stability (WPC only at 5% DH) and overrun (MPC and WPC at all levels of DH) was observed. Foam stability is extensively dependent on the rheological as well as adhesive properties of interfacial film. Poor foam stability can occur due to capillary drainage of lamellae and rapid collapsing of bubbles [29]. The high foam stability at 5% DH can be attributed to the larger molecular weight of released peptides that directly influence foam stability compared to hydrolysates with higher DH and smaller peptides and free amino acids. Similarly, Althouse et al. [29] prepared foams with 5% whey hydrolysate at

pH 7 that showed improved foam capacity (percent overrun). The good foamability of MPC can be attributed to the high flexibility of the casein structure, with similar results being reported by many studies [49–51]. Proteolysis leads to an increase of polypeptide and amino acids content of hydrolysates that enhances the incorporation of air at the air-water interface and thus improves foam capacity [52].

5. Conclusions

Actinidin hydrolysis of MPC and WPC resulted in improvement in certain functional properties. Protein solubility of both substrates increased with increasing DH, with the highest solubility achieved at 15% DH, where especially WPC showed almost full solubility (~97%). Heat stability also increased in the case of WPC only; however, the mixed trend was seen in the case of MPC with maximum heat stability at 5% DH. Despite improvement in solubility and heat stability, both hydrolysed substrates showed poor emulsifying properties compared to these of the intact proteins, with MPC at 15% DH having the lowest EAI among all substrates. Actinidin-induced hydrolysis also improved foaming properties for both substrates, including foaming stability, where MPC with 15% DH had the greatest foam stability of almost double that of the control. The only exception was hydrolysates of whey proteins at 15% DH with a highly compromised foam stability. These results indicate that actinidin can be used to solubilise MPs, thereby improving the functionality of milk proteins (such as solubility, heat stability, foaming stability and overrun) in different foods.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/foods12203806/s1, Figure S1: Second derivative of amide I region (1700–1600 cm^{-1}) of MPH of milk protein concentrate (MPC) and whey protein concentrate (WPC) by actinidin to 0, 5, 10 and 15% DH at 60 °C.

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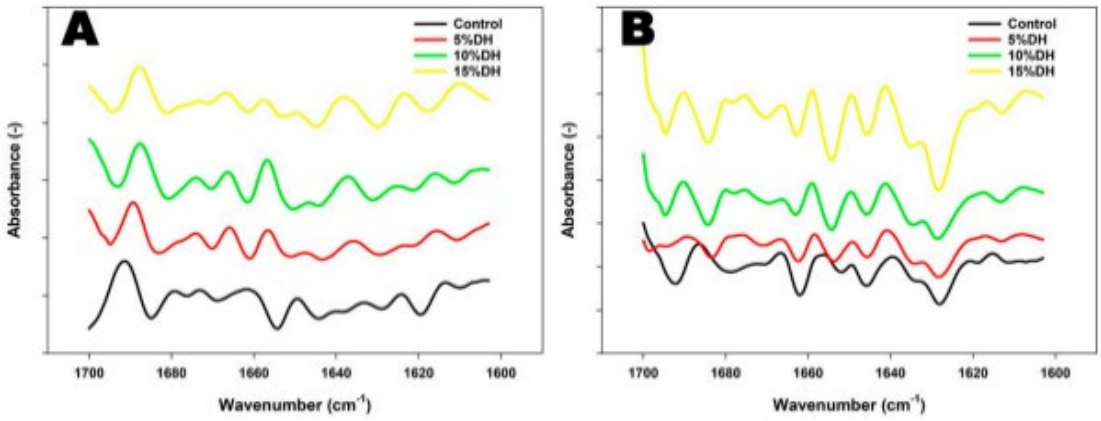
5.2 Supplementary material:

Figure S1: Second derivative of amide I region (1700–1600 cm−1) of MPH of milk protein concentrate (MPC) and whey protein concentrate (WPC) by actinidin to 0, 5, 10 and 15% DH at 60 ◦C.



Chapter 6

Milk Protein Hydrolysis by Actinidin—Kinetic and Thermodynamic Characterisation and Comparison to Bromelain and Papain

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Article

Milk Protein Hydrolysis by Actinidin—Kinetic and Thermodynamic Characterisation and Comparison to Bromelain and Papain

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Abstract: Plant proteases, including actinidin, papain and bromelain, have been widely used in the food industry but with limited application in dairy systems. This research aimed to establish and compare operational parameters (kinetics, temperature, enzyme type, time and thermodynamics) relevant to the applications of these enzymes in the hydrolysis of whey protein isolates (WPI), whey protein concentrates (WPC) or milk protein concentrates (MPC). The degree of hydrolysis (DH) increased with the rise in temperature, and the maximum DH was achieved at 60 °C for all three dairy systems. The addition of papain resulted in a greater %DH of whey proteins in comparison to bromelain. The cleavage of proteins was clearly time-dependent ($p < 0.05$), while the pH did not change significantly ($p > 0.05$) during this time. PAGE analysis revealed that all three enzymes mainly acted on α-lactalbumin and α_s-casein in WPI and MPC, respectively. Kinetic parameters from the Lineweaver–Burk plot at 60 °C using WPC and MPC as a substrate varied widely, establishing that WPC hydrolysis was characterised by a lower KM, higher kcat, kcat/KM and Vmax compared to MPC in the case of all three enzymes. The difference in kcat/KM values amongst all enzymes (actinidin > papain > bromelain) indicated the difference in the strength of substrate binding sites. The thermodynamic parameters of these enzymes with MPC and WPC were also determined at a temperature range of 15–60 °C, and the results indicate the potential application of papain and actinidin in the dairy industry.

Keywords: actinidin; papain; bromelain; proteolysis; milk protein concentrate; whey protein concentrate; whey protein isolate; kinetics; thermodynamic parameters

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1. Introduction

Cysteine proteases (EC.3.4.22), such as actinidin (EC 3.4.22.14), bromelain (EC 3.4.22.32) and papain (EC 3.4.22.2), are plant-based enzymes with a similar substrate specificity, a tertiary structure and amino acid chains [1]. Actinidin contains 220 amino acids and has a molecular weight of 23.5 kDa, bromelain has 285 amino acids and a molecular weight range of 23.4–35.7 kDa and papain has 212 amino acids with a 23.4 kDa molecular weight [1]. These proteases have several advantages, such as mild processing conditions, faster processing rates and economic, health and safety benefits that enable industries to reduce production costs and improve efficiency/productivity [2]. Furthermore, they are also active over a wide range of temperatures and pH. For example, actinidin is active in the pH range 4–10 and between 15 and 60 °C [3,4], bromelain is active at pH 4.6–9.5 and at 10–75 °C [5], and papain is active in the narrower pH range of 5–7 but still remains active at higher temperatures (<90 °C) [2]. Despite having all these common advantages, these enzymes may behave differently even under the same processing conditions; their

thorough kinetic and thermodynamic characterisation is needed to enhance knowledge in this field.

Various studies have shown that these proteases enhance gastric digestion [6], while their impact on the intestinal phase of digestion is rather minor but still noticeable [7,8]. They have been extensively used as meat tenderisers due to their proteolytic actions on collagen and myofibrillar proteins [9–11], as well as beer clarifiers and milk-coagulating agents [12,13]. The proteolytic activity of actinidin has also been used in some dairy systems to minimise antigenicity (allergenicity) and improve functionalities [6,14–16]. Comparatively, however, there is still limited information available for commercial applications of actinidin, bromelain and papain in these dairy systems.

Milk proteins (MP) are widely used in various food products. However, the application of proteins in food systems is sometimes limited due to issues related to allergenicity [17], digestibility [11], solubility or heat stability [18,19]. In some of these cases, the use of protein hydrolysates rather than intact proteins can provide viable solutions. Therefore, the efficient utilisation of milk proteins in food systems depends on tailoring their structural characteristics. Establishing optimal conditions for protein hydrolysis is crucial to obtaining protein hydrolysates with improved structural characteristics [20].

In our previous work, actinidin was used to determine the potential mode of its action and its potential benefits with dairy systems [14,15,21], and this work showed the appreciable effect of actinidin with dairy systems, especially when minimising antigenicity and improving functionalities [14,15]. Therefore, these positive outcomes lead to further exploration of its applications on an industrial basis to develop noble products and processes. To consider industrial applications, however, the development of the process needs to be considered. For this, and to enable adequate process control, the kinetics of hydrolysis reactions are required. Hence, the aim of the current study was to determine the kinetic and thermodynamic parameters of the actinidin-induced hydrolysis of milk proteins. Our second aim was to compare such kinetic and thermodynamic parameters for actinidin with those for the two other aforementioned cysteine proteases, i.e., papain and bromelain. For this purpose, the papain and bromelain-induced hydrolysis of the main proteins in the whey protein isolate (WPI), whey protein concentrate (WPC) and milk protein concentrate (MPC) were studied, and data among those previously reported for actinidin-induced protein hydrolysis in the same ingredients [21] were used to determine appropriate thermodynamic and kinetic parameters. The research question was whether these proteases showed similar behaviour to actinidin as toward the tested milk proteins. The establishment of such a parameter can form an important basis for the design and optimisation of (industrial) processes for the controlled hydrolysis of milk proteins using actinidin, papain or bromelain.

2. Materials and Methods

2.1. Materials

MPC (80%, *w*/*w*, protein on dry matter), WPI (90%, *w*/*w*, protein on dry matter) and WPC (80%, *w*/*w*, protein on dry matter) were sourced from Fonterra Cooperative (Palmerston North, New Zealand). Papain (60,000 activity units mg^{-1}) and bromelain (2200 activity units g^{-1}) were obtained from Connell Bros. Company Australasia (Croydon South, Victoria, Australia). Actinidin (KEP500; 521 activity units g^{-1}) was obtained from kiwiEnzyme.com Ltd. (Martinborough, Wellington, New Zealand). A total of 0.2125 M of sodium phosphate buffer (pH 8.2) and Trinitrobenzenesulfonic acid (TNBS) were used. Simulated milk ultrafiltrate (SMUF) at pH 6.8 was used as a buffer for all samples, including the controls [21]. All other chemicals were sourced from Sigma-Aldrich Pty Ltd (Castle Hill, NSW, Australia).

2.2. Protease-Induced Hydrolysis of Milk Protein Systems

Protein suspensions were prepared at a constant concentration of total solids (5% *w*/*w*) in simulated milk ultrafiltrate (SMUF), as described previously [21]. The protein

suspensions were then equilibrated at a specific temperature (15, 40 or 60 °C) before adding each protease. The pH was not controlled but monitored throughout hydrolysis using a portable pH meter (model 3110 SET2 ProfiLine, Xylem Analytics, Hemmant, QLD, Australia). The same level of the proteases was added based on their activity per unit of mass to facilitate the best comparison among them with all three substrates. The proteases were added at the enzyme-to-substrate ratio (E:S) of 1:100 by adding (5.21 units of each enzyme activity g^{-1} of protein) or 1:500 (1.04 units of actinidin g^{-1} of protein; 1:500 was considered for thermodynamic parameters of actinidin only to compare enzyme efficiency). Subsequently, each protease assay was performed at selected temperatures for 5 h with continuous stirring in a water bath (Grant Instruments, Cambridge Ltd., Barrington, Cambridge, England). An aliquot of 1.0 mL of the hydrolysed samples was taken out on an hourly basis, and then 1 mL of 10% (w/v) SDS was added, followed by heat treatment at 90 °C for 5 min to terminate the reaction. The samples were then stored at −20 °C for further testing.

2.3. Determination of the %DH of Milk Proteins by Papain and Bromelain

As described previously [21], the trinitrobenzenesulfonic acid (TNBS) method [22] was used to determine the number of released peptide bonds by measuring the absorbance of the product formed at a wavelength of 340 nm and using a spectrophotometer (Biochrome Libra S12, Biochrom Ltd., Cambridge, UK). A degree of hydrolysis was calculated on the basis of complete hydrolysis. For this, fully hydrolysed samples were prepared by treating each substrate with 6 M HCl under reflux [23]. In brief, 0.5 g of each sample, with an equivalent protein content to hydrolysates, was obtained via enzymatic hydrolysis, placed in a conical flask with 10 mL of 6M HCl, followed by the addition of 5 to 6 pieces of boiling chips. The test solution was heated at 110 °C in an oil bath in a fume hood under reflux for 24 h. The hydrolysates were then filtered and neutralised, and the absorbance was determined using the TNBS method, as stated previously [21]. A DH was expressed as the percentage of cleaved peptide bonds relative to the completely hydrolysed sample:

$$\%DH = \frac{h}{h - tot} \times 100 \quad (1)$$

where $(h - tot)$ is the total numbers of peptide bonds per protein equivalent obtained from given samples with chemical hydrolysis, and h is the number of hydrolysed bonds.

2.4. Kinetic and Thermodynamic Parameters Describing Milk Protein Hydrolysis by All Three Proteases

For the determination of the kinetic parameters, WPC and MPC suspensions were prepared as per Section 2.2 at protein concentrations of 20, 40, 60, 80 or 100 mg mL^{-1}. Incubations were performed at 60 °C, and reactions were initiated by adding 2.6 units of the enzyme activity g^{-1} of the actinidin, bromelain or papain protein to subsequent samples. During a period of 60 min of incubation, 1 mL of the aliquots were drawn every 5 min after which the reaction was terminated, as described in Section 2.3. The initial velocity at various enzyme-to-substrate ratios by keeping the enzyme constant and varying the substrate, confirmed that data followed the Michaelis–Menten behaviour for both substrates in all three enzymes. Thus, kinetic parameters were established from the equation generated from a linear trendline of the Lineweaver–Burk plot [24].

$$\frac{1}{V} = \frac{KM}{Vmax[s]} + \frac{1}{Vmax} \quad (2)$$

where V is the enzyme velocity (min^{-1}), S is the substrate concentration (μg mL^{-1}), KM is the saturation constant (μg mL^{-1}) and $Vmax$ is the maximum enzymatic activity for substrate conversion (μg mL^{-1} min^{-1}).

For thermodynamic parameters, the hydrolysis process for all enzyme-to-substrate ratios was correlated to the Arrhenius law as follows:

$$k = Ae^{\frac{-Ea}{RT}} \quad (3)$$

where A is known as the pre-exponential factor, also called the frequency factor (1/s), Ea is the activation energy (kJ mol^{-1}), R is the molar gas constant (8.314 kJ mol^{-1}) and T is the treatment temperature in Kelvin.

So, activation energy was calculated using the following equation:

$$Ea = -RT\ln\left(\frac{k}{A}\right) \quad (4)$$

The activation energy (Ea) was calculated from the slope of the Arrhenius plot, graphed utilising the natural log of the reaction rate constant and inverse of temperature in Kelvin at a temperature range of 15–60 °C.

2.5. Protease-Induced Hydrolysis of Milk Proteins

To monitor the hydrolysis of the individual proteins in WPC and MPC, densitometric analysis was performed on SDS-PAGE gels for samples run under reducing conditions (using β-mercaptoethanol), as described previously [21]. The gels were scanned using an imager (Chemidoc MP, Bio-Rad Laboratories, Hercules, CA, USA) and the band quantifications were performed on all the reducing gels in triplicate via Image Lab software (6.0.1 @2017, Bio-Rad Laboratories Inc., Hercules, CA, USA).

2.6. Statistical Analysis

All experiments, including hydrolysis and kinetics, were replicated at least three times for all three substrates. The data obtained were analysed as a split-plot design repeated in time measurements with the SAS software (ver. 9.1) and the GLM protocols. Then, the means were compared using the LSMEANS function with the option of PDIFF, and the significance was set at $p < 0.05$.

3. Results

3.1. Impact of Processing Conditions on pH and DH

The change in pH during the proteolysis of MPs with bromelain and papain was monitored throughout the incubation. The decline in pH after 5 h, relative to the controls, is shown in Table 1. From the table, three clear trends can be seen, i.e., (1) in the case of both enzymes, an increase in incubation temperature resulted in a greater decline in the pH; (2) in the case of bromelain, greater a pH decline was observed upon WPC or WPI hydrolysis than that of MPC, particularly at 40 and 60 °C; and (3) in contrast to bromelain, papain activity resulted in a more prominent decrease in pH after the hydrolysis of MPC compared to WPC or WPI and in particular at 60 °C. The greatest decline in pH of 0.44 (bromelain) and 0.35 (papain) was observed at 60 °C for WPI and MPC, respectively (Table 1). The change in pH and %DH for actinidin during the 5h incubation was already reported previously when the maximum pH decline was 0.57 (MPC) and 0.28 (WPI and WPC) at 60 °C [21].

In the current study, the %DH for all samples increased with the increase in the incubation temperature (15 to 60 °C) (Table 2). These results are in line with the previous study in which hydrolysates obtained with actinidin showed the same trend [21]. The %DH of actinidin, obtained under the same conditions, is not included in the current study as it is already reported [21]. Furthermore, both substrate suspensions incubated above 60 °C underwent a sol-gel transition via both bromelain and papain and, thus, could not be analysed further. Notably, %DH did not plateau during the incubation time (5 h). Furthermore, the %DH varied widely between substrates during the determined time and ranged from ∼6 to ∼17%. In the case of the hydrolysates obtained using both bromelain and papain at 60 °C, WPI showed the greatest %DH followed by WPC, whereas MPC

showed the lowest %DH (Table 2). Interestingly, whey protein-based ingredients were hydrolysed with papain and reached almost double the %DH as that of bromelain after 5 h of incubation at 60 °C.

Table 1. The pH decline (final–initial) after 5 h of incubation of suspensions (5% *w*/*w*) of MPC, WPC or WPI with papain or bromelain at an enzyme-to-substrate ratio of 5.21 units g^{-1} and a temperature of 15, 40 or 60 °C.

Temp (°C)	Papain			Bromelain		
	MPC	WPC	WPI	MPC	WPC	WPI
15	0.02 ^{aA}	0.02 ^{aA}	0.08 ^{aB}	0.12 ^{aC}	0.12 ^{aC}	0.13 ^{aC}
40	0.19 ^{bBC}	0.17 ^{bB}	0.18 ^{bB}	0.14 ^{abA}	0.15 ^{abA}	0.23 ^{bD}
60	0.35 ^{cE}	0.26 ^{cC}	0.30 ^{cD}	0.17 ^{bcA}	0.21 ^{cB}	0.44 ^{cF}
SEM	0.01					

SEM—pooled standard error of the mean; upper-case and lower-case superscript letters display significant difference ($p < 0.05$) within a row and a column, respectively.

Table 2. Degree of hydrolysis (%DH) reached after 5 h of incubation of (5% *w*/*w*) with suspensions of MPC, WPC or WPI with papain or bromelain at an enzyme-to-substrate ratio of 5.21 units g^{-1} and a temperature of 15, 40 or 60 °C.

Degree of Hydrolysis (%)				
Enzyme	Temperature (°C)	Substrate		
		MPC	WPC	WPI
Bromelain	15	1.67 ^{fC}	2.89 ^{fB}	3.45 ^{fA}
	40	3.82 ^{dC}	6.42 ^{eB}	7.12 ^{eA}
	60	6.45 ^{bC}	8.96 ^{cB}	9.23 ^{cA}
Papain	15	3.23 ^{eC}	6.9 ^{dB}	8.0 ^{dA}
	40	5.38 ^{cC}	11.53 ^{bB}	12.57 ^{bA}
	60	8.12 ^{aC}	16.77 ^{aB}	17.69 ^{aA}
SEM		0.03		

Values are the means of at least 3 independent observations ($n \geq 3$); SEM = standard error of the mean. The means in the same column displayed by different capital letter superscripts are significantly different ($p < 0.05$). The means in the same row displayed by different small letter superscripts are significantly different ($p < 0.05$).

3.2. Proteolysis Patterns for Enzymatic-Induced Hydrolysis of MPC and WPC

The results from monitoring the hydrolysis of individual milk proteins in MPC and WPC via the densitometric analysis of SDS-PAGE gels are shown in Table 3. The patterns of protein hydrolysis were compared and they appeared to be in agreement with the %DH achieved. A substantial reduction in the band intensities was aligned with an increase in the temperature for all samples. Papain showed the greatest band intensity reduction, followed by actinidin and bromelain, respectively. Even the level of MPC hydrolysis with bromelain did not differ much in proportion to the remaining proteins at 15 and 40 °C (Table 3). Of the caseins, the α_s-CNs appeared more susceptible to hydrolysis than β- or κ-CN, particularly for actinidin and papain (Table 3). As opposed to these observations, bromelain exerted a similar cleavage action on all proteins after 5 h of hydrolysis. For example, for α_s-, β- or κ-CNs, the proportion of the remaining protein fractions ranged between ~60 and 70% compared to the control. The hydrolytic action of all three enzymes on whey proteins in MPC appeared to be directed at α-LA as β-LG was not affected as much. The trend appeared unchanged with the change in the substrate since actinidin and papain action exerted their activity mainly on α-LA and, to a lesser extent, on β-LG when the WPC was used as a substrate. This was most apparent at 60 °C, with ~16% and

~9% of β-LG and ~6% and ~5% of α-LA remaining after 5 h of incubation with actinidin and papain, respectively. Bromelain, however, exerted the low and equal cleavage of these proteins with about 38% of β-LG and ~36% of α-LA remaining (Table 3).

Table 3. Proportion of milk proteins (%) remaining after hydrolysis of MPC (5% *w*/*w*) or WPC (5% *w*/*w*) with actinidin, bromelain or papain at 60 °C for 5 h. The proportion expressed is relative to the un-hydrolysed control estimated from the reduced SDS-PAGE gels.

Protein	Proportion of Proteins Remaining Relative to Control (%)								
	Temperature (°C)								
	15	40	60	15	40	60	15	40	60
MPC	Proteases								
	Actinidin			Bromelain			Papain		
α_s-CN	86.0 ^{bB}	31.0 ^{fD}	16.6 ^{hE}	88.7 ^{aB}	85.7 ^{cA}	72.5 ^{dA}	65.3 ^{eC}	17.7 ^{gD}	9.3 ^{iC}
β-CN	87.4 ^{aA}	28.4 ^{fE}	22.1 ^{gB}	70.5 ^{bG}	66.2 ^{cE}	64.3 ^{dC}	49.0 ^{eF}	15.5 ^{hF}	10.2 ^{iB}
κ-CN	75.9 ^{bE}	25.3 ^{fG}	17.8 ^{gC}	78.5 ^{aD}	72.0 ^{cD}	66.5 ^{dB}	62.7 ^{eD}	10.5 ^{hG}	10.2 ^{iB}
β-LG	83.5 ^{aC}	40.4 ^{fB}	25.1 ^{hA}	81.8 ^{bC}	75.5 ^{dC}	72.4 ^{eA}	78.0 ^{cA}	26.7 ^{gB}	19.6 ^{iA}
α-LA	80.6 ^{aD}	45.5 ^{fA}	17.0 ^{hD}	77.3 ^{bE}	76.8 ^{cB}	57.0 ^{eD}	68.5 ^{dB}	32.7 ^{gA}	10.2 ^{iB}
WPC	Actinidin			Bromelain			Papain		
β-LG	48.2 ^{bF}	34.2 ^{eC}	16.2^{hF}	76.8 ^{aF}	42.0 ^{cF}	37.7 ^{dE}	28.4 ^{fG}	19.3 ^{gC}	8.9 ^{iD}
α-LA	47.8 ^{cG}	26.3 ^{fF}	6.1^{hG}	94.1 ^{aA}	37.6 ^{dG}	35.5 ^{eF}	54.0 ^{bE}	17.2 ^{gE}	5.3 ^{iE}

The standard error of the mean (SEM) of at least three independent observations for hydrolysates of MPC and WPC was 0.191 and 0.192, respectively; lower- and upper-case superscript letters indicate significant difference ($p < 0.05$) within a row and a column, respectively.

3.3. Thermodynamic Characterisation of Actinidin, Bromelain and Papain

The thermodynamic parameters for actinidin, bromelain and papain-induced hydrolysis of milk proteins were established from the Arrhenius plot. Table 4 depicts the dependence of ln *k* with 1/*T* for hydrolysis reactions at various temperatures and incubation times for each protease. In the case of all three enzymes, the Arrhenius plot was fitted with linear functions with a coefficient of correlation ranging from 0.81 to 0.99. At an E:S of 1:100 (5.21 Units g^{-1} of protein), the activation energy of bromelain with MPC was the greatest, whereas papain exhibited the lowest Ea with WPC. The activation energy (Ea) for MPC, WPC and WPI was found to be about 25.4, 23.9 and 24.5 kJ mol^{-1} (for bromelain) and 13.68, 12.07 and 11.05 kJ mol^{-1} (for papain), respectively. Moreover, the activation energy (Ea) for MPC, WPC and WPI with actinidine was found to be 18.1, 15.0 and 14.4 kJ mol^{-1}, respectively. When the E:S ratio changed to 1:500 (1.04 Units g^{-1} of protein), the activation energy (Ea) for MPC, WPC and WPI changed to 21.1, 17.1 and 15.3 kJ mol^{-1}, respectively (Supplementary Materials, Table S1). The highest activation energy reported was about 25 kJ mol^{-1} in the case of MPC with bromelain, which is far lower than that reported for different proteases and/or substrates. For example, a study with ficin protease reported an activation energy of about 54 kJ mol^{-1} using casein as a substrate [25]. An activation energy of around 74 kJ mol^{-1} was noted for papain using collagen as a substrate [26].

Table 4. Reaction constants (k), coefficient of correlation (r^2), Arrhenius activation energies (E_a) and pre-exponential factors (A) established from the Arrhenius plots for the hydrolysis of proteins in 5% (w/w) suspensions of MPC, WPC or WPI with actinidin, bromelain or papain at an enzyme-to-substrate ratio of 5.21 units g^{-1} of protein and a temperature range between 15 and 60 °C.

Enzyme	Substrate	Temp °C	K ($\times 10^{-6}$, s^{-1})	r^2 (1)	Ea (kJ mol^{-1})	A	r^2 (2)
Actinidin (5.21 Units g^{-1} of protein)	MPC	15	3.46	0.97	18.09	0.0079	0.81
		35	8.66	0.97			
		40	9.14	0.94			
		55	9.71	0.97			
		60	9.99	0.93			
	WPC	15	6.98	0.94	15.02	0.0037	0.99
		35	10.07	0.9			
		40	11.62	0.91			
		55	15.37	0.89			
		60	15.77	0.9			
	WPI	15	6.78	0.89	14.38	0.0028	0.98
		35	10.54	0.9			
		40	11.84	0.9			
		55	14.49	0.89			
		60	15.15	0.89			
Bromelain (5.21 Units g^{-1} of protein)	MPC	15	0.4	0.96	25.39	0.0158	0.99
		40	0.93	0.97			
		60	1.65	0.92			
	WPC	15	0.49	0.81	23.91	0.0112	0.96
		40	1.33	0.99			
		60	1.86	0.94			
	WPI	15	0.75	0.78	24.52	0.0009	0.98
		40	1.47	0.98			
		60	1.92	0.96			
Papain (5.21 Units g^{-1} of protein)	MPC	15	0.63	0.99	13.68	0.0002	0.97
		40	0.9	0.96			
		60	1.38	0.99			
	WPC	15	1.43	0.95	12.07	0.0002	0.99
		40	2.04	0.98			
		60	2.83	0.98			
	WPI	15	1.65	0.99	11.05	0.0002	0.96
		40	2.16	0.95			
		60	3.11	0.97			

(1) Coefficient of determination for k; (2) Coefficient of determination for Ea.

3.4. Estimation of Kinetic Parameters for Milk Protein Hydrolysis by Actinidin, Bromelain and Papain at 60 °C

The kinetic parameters, including Vmax, $\frac{1}{2}$Vmax, kcat, KM and kcat/KM were determined for all three enzymes using the Lineweaver–Burk plots and MPC and WPC as the

substrates (Table 5). The reciprocal plot of the initial velocity versus substrate concentrations showed very good linearity, providing r^2 = 0.99 and 0.97 (for actinidin), 0.97 and 0.96 (for bromelain) and 0.96 and 0.93 (for papain) for MPC and WPC, respectively. The Michaelis–Menten constant (KM) was significantly ($p < 0.05$) different between the substrates but showed a consistent trend among the enzymes (papain < actinidin < bromelain).

Table 5. Michaelis–Menten kinetic parameters describing the hydrolysis of the milk protein concentrate (MPC) and whey protein concentrate (WPC) suspensions (5% *w*/*w*) using actinidin, bromelain or papain (2.6 units of enzyme activity g^{-1}) with substrate concentrations of 20, 40, 60, 80 or 100 mg mL^{-1} determined at 60 °C.

Enzyme	Substrate	Vmax	1/2 Vmax	KM	Kcat	Kcat/KM
		(μg mL $^{-1}$ min^{-1})	(μg mL^{-1} min^{-1})	($\times 10^{-4}$, μg mL^{-1})	(min^{-1})	(mL min^{-1} μg^{-1})
Actinidin	WPC	3.96	1.98	2.13	0.99	46
	MPC	1.56	0.78	3.18	0.39	12
Bromelain	WPC	1.29	0.65	10.71	0.32	3
	MPC	1.01	0.50	17.71	0.25	1
Papain	WPC	1.12	0.56	1.87	0.28	15
	MPC	1.28	0.64	3.05	0.32	11

The lower the KM, the higher the binding affinity of the protease with a particular substrate [27]. In the current study, the lowest KM values were obtained for papain with WPC and MPC at about 1.87 × 10^4 and 3.05 × 10^4 μg mL^{-1}, respectively. Actinidin had a slightly higher KM with WPC (2.13 × 10^4 μg mL^{-1}) and MPC (3.18 × 10^4 μg mL^{-1}) than papain but was still much lower compared to that of bromelain. Bromelain was characterised as having the highest KM (μg mL^{-1}) amongst all the enzymes of 10.71 × 10^4 and 17.71 × 10^4 for WPC and MPC, respectively (Table 5).

With WPC as the substrate, all three proteases were characterised by a low KM value (above stated), a high turnover number (kcat) and high kcat/KM (mL min^{-1} μg^{-1}) in comparison to those of MPC (Table 5). These results indicate that all enzymes acted preferentially on the peptide bonds of WPC. Clearly, WPC appears to be a better substrate in comparison to MPC. For example, a turnover number (kcat) of WPC with papain, actinidin and bromelain (0.28, 0.99 and 0.32 min^{-1}, respectively) was greater than that of MPC (0.32, 0.39 and 0.25 min^{-1}). Also, kcat/KM values for WPC with papain, actinidin and bromelain (15, 46 and 3mL min^{-1} μg^{-1}) were much greater than that of MPC (11, 12 and 1 mL min^{-1} μg^{-1}) (Table 5).

4. Discussion

Operational parameters such as the incubation temperature, pH, substrate type and reaction time are very important variables in order to establish the feasibility of applications of any enzyme, including actinidin, bromelain and papain, in the industry. After obtaining the operational conditions for these proteases with milk protein substrates (Tables 1 and 2), the thermodynamic (Table 4) and kinetic (Table 5) parameters were determined using WPC and MPC as the substrates to characterise or establish a relevant knowledge base in relation to their enzyme activity. Proteolytic patterns used SDS-PAGE to confirm the degree of hydrolysis of milk proteins (Table 3).

The current study was conducted without a pH adjustment in order to replicate commercial conditions. The pH reduction took place, but it remained in the range of 6.73–5.99 throughout the process for all enzymes and substrates (Table 1). Overall, the pH change was slight, with papain achieving a greater change in pH at a maximum incubation temperature and time than bromelain, except for WPI. Neutralisation (pH control) usually results in a high salt content, which may the limit implementation of these hydrolysates in

certain applications; thus, achieving the required level of hydrolysis without pH control is desirable from an industrial point of view. However, the change in pH may impact the structural confirmation of the enzyme and, thus, its activity, leading to the altered composition of released peptides [28].

Our current study showed how %DH increased with an elevation in incubation temperature and time (Table 2). These results concurred with the results obtained from our previous study [21]. Also, in the current study, papain demonstrated similar proteolytic activity to actinidin, while bromelain appeared to be vastly different. For example, whey protein hydrolysates obtained using bromelain reached only around 9 %DH at 60 °C, whereas papain showed double (around 18 %DH) after 5 h of incubation (Table 2). This clearly explains the difference in the specificity of these proteases towards these substrates despite being categorised in the same CA family group. So, papain has the potential to be used in certain applications where a high DH is required to modify the protein structure to achieve, e.g., hypoallergenicity or functionality. Al-Shamsi, Mudgil, Hassan, and Maqsood [29] also showed that the maximum %DH of camel milk protein hydrolysates obtained using papain (∼ 40%) was almost double that achieved by bromelain (∼ 24%) after 6 h under the same processing conditions.

Papain has Cys_{25} and His_{159} present in its active site, and it preferentially cleaves Ala, Ile, Trp, Phe, Val, Leu and Tyr amino acid residues with the peptide bonds of hydrophobic regions. Papain exhibits a preference for an amino acid with a large hydrophobic side chain at the P2 position; however, it does not accept Val at P1's position [30]. In the case of actinidin, Cys_{25} and His_{162} are two residues present at the active site behind the cleft in the middle of the domains, and they bind with the substrate to create an enzyme–substrate complex. Actinidin cleaves Phe, Val and Leu. P1's position is a specificity determinant, and, unlike papain, this enzyme can accept Val at P1's position [31,32]. However, in the case of bromelain, it has limited substrate specificity, and it only cleaves the carbonyl end of Lys, Ala, Tyr and Gly [32].

For MPC, β-CN has a hydrophobic region at the C-terminus of 136–209 residues [33,34]. This sequence contains all three Leu (10), Val (8) and Phe (3) target amino acid residues that are essential for actinidin to act on. For papain, including all three amino acids, the other four amino acids, Ala (2), Ile (3), Trp (1) and Tyr (2), are also present as potential cleavage sites. On the other hand, in the case of bromelain, a very limited number of amino acids (two residues of each Lys, Ala and Tyr and one Gly) is available. For this reason, a greater possibility exists for papain and actinidin to cleave these proteins, resulting in a reduction in the molecular weight of proteins/peptides and an increased %DH, which is also reflected in the results (Table 2). Similarly, in the case of αs_1-CN, the amino acid fraction from 100 to 199 is considered a highly hydrophobic region present at the carboxyl-terminal [33], which consists of Leu (6), Val (3) and Phe (4) as potential target sites for actinidin and papain. For papain, the other four amino acids, including Ala (5), Ile (5), Trp (2) and Tyr (8), are also present in the primary structure of αs_1-CN as possible targets. Also, in the same protein fraction, Ala (5) and Tyr (8), Lys (6) and Gly (5) are present and are potential cleavage sites for bromelain to act on. Furthermore, in the case of αs_2-CN, the amino acid chain of 126 to 207 is considered a highly hydrophobic region [33]. This region contains Leu (5), Val (5), Phe (3) (potential cleavage sites of actinidin and papain) and Ala (2), Ile (3), Trp (1) and Tyr (4) (more cleavage sites for papain to bind with). Whereas in the case of bromelain, despite the presence of adequate amounts of Lys (14), there is still an absence of Gly and with only limited amounts of Ala (2) and Tyr (4) [32], which could contribute to lower proteolysis compared to papain and actinidin (Table 2), as reflected in Table 3.

When it comes to major whey proteins, β-LG contains two disulphide bridges located at Cys_{106}-Cys_{119} and Cys_{66}-Cys_{160} that contribute to the stability of this protein [35,36]; however, the cleavage of these disulfide bonds by these proteases can lead to an enhanced susceptibility to hydrolysis [37]. Therefore, actinidin and papain (due to Cys present at their active sites) could have cleaved these disulphide bonds, resulting in the disappearance of bands in the PAGE gels (Table 3) and an overall increment in %DH (Table 2). On the other

hand, the cleavage of disulphide bonds is not supported by bromelain [32], and the SDS PAGE of the current study also resulted in the presence of a higher β-LG-remaining protein fraction compared to papain and actinidin (Table 3). α-LA, as proportionally the second most abundant whey protein, is characterised by the presence and abundance of Leu (14), Val (6) or Phe (4) [38], which allows for the greater binding of papain and actinidin with more random action than bromelain, which may contribute towards greater proteolysis (Table 3). The remaining protein representing α-LA in Table 3 disappeared at a greater rate than those of β-LG, which indicated a greater affinity towards α-LA than β-LG.

Limited proteolysis results in hydrolysates with a low level of DH and is frequently desirable in these cases when improvements of a specific functionality are needed, such as improved emulsifying properties or foam stability. Such modifications assist with the structuring of the final products, such as processed meats, edible films, ice creams, froth drinks and salad dressings [39]. Bromelain, despite showing a very low %DH under the current processing conditions (Table 2), can still be used in applications where small but targeted hydrolysis may be needed, such as an improvement in certain functional properties. For example, salad dressing prepared using 1 %DH whey protein hydrolysates resulted in a creamy texture in the final product compared to the control, which was prepared using unhydrolysed WPC and where the final product appeared very runny with a thin texture. A further slight increase in %DH (remaining between 2–4%) resulted in dressings with desirable stability [40].

It is well known that hydrolysis can proceed at a greater rate if the activation energy is low [41]. The activation energy of actinidin (at both substrate ratios varied between 14 and 17) and papain (∼11–12) for whey proteins appeared substantially lower than those calculated for bromelain (between 23–24) (Table 4). This also supports our results by indicating the range of enzyme efficiency in dairy systems in the following order: papain > actinidin > bromelain.

Also, kinetic parameters (Table 5) are in agreement with the results obtained for %DH, where all three proteases showed a greater cleavage of the peptide bonds in the case of WPC than MPC. Bromelain showed the lowest cleavage action in the case of both substrates compared to actinidin and papain (Table 2). Proteases such as aspartic [42,43] and serine [25] with various substrates obey the Michaelis–Menten kinetics. Only these enzymes show this behaviour and have the ability to bind with a specific substrate due to their specific active sites rather than allosteric sites [25]. A study by Salami et al. [44] showed that a serine protease also showed Michaelis–Menten behaviour during the hydrolysis of milk proteins, including WPC and caseins. The KM values for casein and WPs with chymotrypsin were 0.81 and 3.38 mg mL^{-1}, respectively, and with trypsin, 3.78 and 1.37 mg mL^{-1}, respectively. In comparison to the current study (Table 5), the addition of actinidin and papain to WPC and MPC suspensions resulted in KM of 2.13×10^4 to 3.18×10^4 and 1.87×10^4 to 3.05×10^4 (Table 5), which is equivalent to 21.3 to 31.8 and 18.7 to 30.5 mg mL^{-1}, respectively. However, in the case of bromelain, KM values were about 5 and 6 times greater (WPC and MPC, respectively) than papain and actinidin, which showed their lowest binding affinity compared to the other two proteases. The specificity and activity of an enzyme depends on the substrate and mode of action despite, for example, the fact that these three enzymes belong to the same family and share similar structure and functions [45]. The difference in kcat/KM values amongst all the enzymes suggests that there is a difference between these three enzymes in the strength of substrate binding sites. These *Kcat* differences can contribute to the binding strength [1]. Therefore, the weaker binding of substrates in the productive mode towards papain can result in lower kcat despite having the lowest KM. Kinetic and thermodynamic studies are important for the greater control of reaction rates and are a clearly understood reaction mechanism to optimise the hydrolysis process [25]. Therefore, the optimisation of processing conditions by understanding changes in milk protein conformations and evaluating kinetic and thermodynamic parameters can lead to knowledge of the more appropriate selection of a particular enzyme to achieve beneficial properties, such as changes in functional properties,

digestibility or alterations in allergenicity [2]. An example of this is if industries are after obtaining a final product at a faster rate, but thermal stability is not required such as certain products manufactured at lower temperatures. In this situation, kinetics in terms of a lower KM is an important parameter to choose instead of activation energy. However, where a reaction at a faster rate is required at a certain range of temperatures, both thermodynamic and kinetic data provide useful information for the selection of protease/s.

5. Conclusions

As this study shows, these three proteases did not express the same proteolytic behaviour toward milk proteins. Papain showed the greatest %DH with whey proteins (∼18% DH), whereas bromelain showed the lowest (∼7%DH) with MPC at 60 °C after 5 h of incubation. The decline in pH was temperature-dependent, with the highest difference of around 0.35 obtained during the hydrolysis of MPC with papain and 0.44 during WPI hydrolysis with bromelain at 60 °C. The SDS-PAGE results revealed α_s-casein and α-LA were preferential substrate fractions in the case of MPC and whey, respectively. Kinetic and thermodynamic parameters revealed that papain and actinidin behaved similarly and showed greater substrate specificity compared to bromelain. Papain showed the lowest KM, whereas bromelain showed the highest KM value. The kcat/KM values followed an actinidin > papain > bromelain order. WPC was a preferential substrate for all three proteases. Also, the activation energy of papain was lowest, and bromelain was highest. These results indicate that actinidin and papain exert appreciable activities toward dairy proteins and have the ability to be used in certain products where specific characteristics are required. However, further knowledge, such as the sequencing of released peptides, can surely add light to evaluate the behaviour of these enzymes toward a specific substrate.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/foods12234248/s1. Table S1: Reaction constants (k), coefficient of correlation (r^2), Arrhenius activation energies (E_a) and pre-exponential factors (A) established from the Arrhenius plots for the hydrolysis of proteins in 5% (w/w) suspensions of MPC, WPC or WPI with actinidin at an enzyme-to-substrate ratio of 1.04 units g^{-1} of protein and a temperature range between 15 and 60 °C.

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6.2 Supplementary material

Table S1: Reaction constants (*k*), coefficient of correlation (r^2), Arrhenius activation energies (E_a) and pre-exponential factors (A) established from the Arrhenius plots for hydrolysis of proteins in 5% (w/w) suspensions of MPC, WPC or WPI with actinidin at an enzyme to substrate ratio of 1.04 Units g^{-1} of protein and a temperature range between 15 and 60°C.

Enzyme	**Substrate**	**Temp °C**	***K*** **(x10^{-6}, s^{-1})**	**r^2 (1)**	**E_a** **(kJ mol^{-1})**	**A**	**r^2 (2)**
Actinidin (1.04 Units g^{-1} of protein)	MPC	15	3.95	0.95	21.07	0.0273	0.97
		35	8.38	0.92			
		40	9.3	0.96			
		55	11.88	0.94			
		60	13.63	0.98			
	WPC	15	5.37	0.87	17.14	0.0068	0.99
		35	9.2	0.87			
		40	9.19	0.89			
		55	12.93	0.86			
		60	14.18	0.86			
	WPI	15	6.1	0.92	15.28	0.0034	0.98
		35	9.14	0.95			
		40	10.07	0.93			
		55	13.35	0.93			
		60	14.63	0.87			

Chapter 7

Conclusions and Future Directions

7.1 Overall conclusions

The aim of the research was to establish the optimal performance of plant proteases for the partial or complete hydrolysis of reconstituted milk protein preparations to achieve enhanced hypo allergenicity or functionality. Therefore, three main objectives were proposed and achieved. The first objective established the relevant conditions (temperature, E:S, time) for optimum hydrolytic activity of a commercial protease (actinidin) using milk protein preparations as substrates. The degree of hydrolysis was determined using the TNBS assay at E:S ratio of 1:100 and 1:500 and at 15, 35, 40, 55 and 60 °C for all three substrates (MPC, WPC and WPI). It was found that actinidin appears to prefer whey proteins over caseins, and the extent of hydrolysis established by %DH was clearly temperature dependant and peaked at 60 °C. In the case of whey proteins, a decrease in the E:S of ratio from 1:500 to 1:100 increased the %DH significantly. The extent of MPC hydrolysis, however, was not affected by changing the enzyme concentration. Overall, actinidin exerted an appreciable and specific activity towards dairy proteins as substrates. The knowledge generated from first objective was implemented in the second stage in order to determine the extent and specificity of proteolysis in relation to expression of allergenicity.

For the second objective, the enzyme (actinidin) was added to MPC & WPC at the previously determined E:S ratio, and the proteolysis performed under predetermined optimum conditions (temperature, pH, time). The temperature of 10 °C was also selected for its potential to lower hydrophobic interactions, which result in diffusion of some individual caseins out of the micelle and likely conformational changes to the proteins. After hydrolysis, the allergenicity of the hydrolysates was determined on the basis of β-LG and αs_1-CN capacity to bind with antibodies using ELISA quantification kits. It was found that both substrates showed enhanced %DH at 60 °C than at 10 °C. A higher %DH resulted in greater reduction in antigenicity due to the cleavage of conformational epitopes, thus resulting in a reduction of the binding capacity

of specific antibodies. Although actinidin was able to hydrolyse αs_1-CN at both temperatures, it was relatively ineffective at lower temperatures for β-LG in WPI. However, at 60 °C it showed the %DH of WPI and a significant reduction in residual antigenicity. Overall, milk proteins hydrolysates with actinidin at 60 °C exerted an appreciable effect in antigenicity reduction.

Further functional properties (solubility, heat stability, foaming and emulsification ability) of various milk protein preparations were then determined under the third objective. The various samples (at predetermined conditions) were hydrolysed using actinidin at different time intervals until 0, 5, 10 and 15% DH was achieved for each substrate. The results revealed that significant changes in the functionality of MPH are associated with %DH. The solubility of MPH increased with increasing %DH where whey proteins attained more than 92% solubility. The PAGE analysis revealed that the most soluble proteins were α-lactalbumin and κ-casein in WPC and MPC, suggesting greater susceptibility to the actions of actinidin under the test conditions. Furthermore, both substrates showed poor emulsifying properties compared with the untreated MPC and WPC, whereas the heat stability, foaming properties and foam stability improved for both substrates. The one exception to this finding was in the case of whey protein hydrolysates at 15% DH. Overall, actinidin can be used to solubilise MPs, thereby improving their functionality (such as solubility, heat stability, foaming stability and overrun) in different foods.

The three objectives of this study sequentially succeeded by: optimising the processing conditions of milk protein preparations (first objective); demonstrating that the optimised conditions corelated with the minimisation of allergenicity (second objective); and enhancing the functionality by optimising solubility, heat stability, emulsification and foaming (third objective).

7.2 Future directions

The findings of the current research have revealed that actinidin exerted an appreciable and specific activity towards milk protein preparations with the processing parameters (temperature, E:S ratio and time) relevant for optimal performance of actinidin successfully established. Although this research was conducted at uncontrolled pH, there were no significant changes in pH noted throughout the process. Further studies are required to establish the relationship between uncontrolled and controlled pH and its effect on other properties of hydrolysates.

Moreover, research could be performed to explore the influence of other environmental factors on MPH improvements such as the combination of enzymatic hydrolysis with high pressure treatment, the use of more than one enzyme, or microwave technology.

One of the interesting findings was the observation that proteolysis can reduce the antigenicity of β-LG and αs_1-CN to a certain extent in milk protein systems. It would be important for future supplement treatments developed using enzymatic hydrolysis to inhibit immunogenic reactions more completely. In addition, the antigenicity determination of other allergenic fractions such as α-LA and αs_2-CN could also add valuable insight to the field. Moreover, the present study determined immunogenicity *in vitro* which cannot be directly compared with the complex human immune system. Thus, *in vivo* studies are recommended to fully evaluate the immunogenicity of the resulting products.

As observed in this study, MPH possesses poor emulsifying capabilities although other functionalities are improved. Further work such as rheological and imaging studies would be a useful approach in understanding the behaviour of the hydrolysates. Additionally, further manipulation of the hydrolysis parameters and processing conditions could improve the functionality of MPH.

Actinidin was compared with other proteases (bromelain and papain) in terms of their kinetic and thermodynamic characteristics, with papain showing a similar behaviour to actinidin whereas bromelain was not as effective. There are countless other plant-based proteases that could be further explored and optimised for use in dairy systems.

Overall, further similar studies are needed with objectives including enzyme selection and the establishment of optimal processing conditions to provide valuable knowledge to the milk processing industries. Protocols for the processing of milk proteins that would be less allergenic and more functional could be established and validated. This would ultimately provide both the CMPA affected and general population with a range of valuable amino acids sources while minimising the risks of allergies.