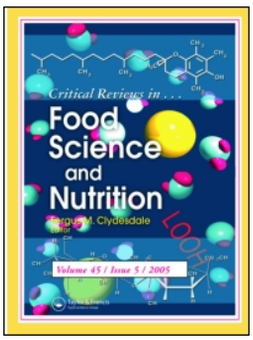

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Impact of the environmental conditions and substrate pre-treatment on whey protein hydrolysis: A review

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ABSTRACT

Proteins in solution are subject to myriad forces stemming from interactions with each other as well as with the solvent media. The role of the environmental conditions, namely pH, temperature, ionic strength remains under-estimated yet it impacts protein conformations and consequently its interaction with, and susceptibility to, the enzyme. Enzymes, being proteins are also amenable to the environmental conditions because they are either activated or denatured depending on the choice of the conditions. Furthermore, enzyme specificity is restricted to a narrow regime of optimal conditions while opportunities outside the optimum conditions remain untapped. In addition, the composition of protein substrate (whether mixed or single purified) have been underestimated in previous studies. In addition, protein pre-treatment methods like heat denaturation prior to hydrolysis is a complex phenomenon whose progression is influenced by the environmental conditions including the presence or absence of sugars like lactose, ionic strength, purity of the protein, and the molecular structure of the mixed proteins particularly presence of free thiol groups.

In this review, we revisit protein hydrolysis with a focus on the impact of the hydrolysis environment and show that preference of peptide bonds and/or one protein over another during hydrolysis is driven by the environmental conditions. Likewise, heat-denaturing is a process which is dependent on not only the environment but the presence or absence of other proteins.

KEYWORDS

Whey proteins; α-lactalbumin; β-lactoglobulin; pH-stat; selective hydrolysis; Acid Protease A

General introduction

Enzyme technology has expanded over time from the basic cheese-making and alcohol fermentation technologies to other sectors including production of value-added products, fine chemicals and pharmaceuticals. The main driving forces for choice of enzyme processes are its sustainability and versatility in addition to being able to proceed under mild environmental conditions devoid of the production of toxic by-products associated with chemical-mediated processes. In addition, with expansion of precise molecular level knowledge about how enzymes work, it is has become increasingly possible to cut down on their wastage enzymes, increased utilization of the biomass with little or no waste materials as well as the ease to scale-up in order to achieve mass-production of safe products. In addition, the body of knowledge on enzyme-substrate specificity has led to improvements in the targeting of enzyme applications and achievement of predictable products. This has led to dramatic cost-cutting procedures with environmentally friendly impacts.

Although there are many applications of enzyme processes in the food industry, hydrolysis of proteins is perhaps one major application. Enzyme hydrolysis of proteins is a competitive area in enzyme technology because of the exciting new products resulting thereof. Apart from conversion to entirely new products, hydrolysis of proteins has been acclaimed to confer benefits to the hydrolysates over that of the parent protein. These include, among others improved functional (Chobert et al., 1988; Lieske and Konrad, 1996; de Wit, 1998; Ipsen et al., 2001; Wasswa et al., 2007), bioactive properties (Gill et al., 1996; Silva and Malcata, 2005), sensory properties (Spellman et al., 2009) as well as low allergenicity (Mahmoud et al., 1992; Exl and Fritsche, 2001) and immunogenicity (Chirico et al., 1997). Regarding functional properties, better solubility of the hydrolysates at low pH compared to the parent proteins from which they are obtained was reported by several workers (Adler-Nissen, 1976; Chobert et al., 1988; Wagner et al., 2000).

Food has increasingly become important as a disease-controlling agent and in that regard the emergence of interest in bioactive peptides obtained from food proteins during fermentation (Takano, 2002; Hernandez-Ledesma et al., 2004) or after deliberate enzyme hydrolysis (Hernández-Ledesma et al., 2006; Ferreira et al., 2007; Kim et al., 2007b) opened new frontiers that elevated food proteins from basic nutrients to functional foods. Therefore, cheese (Didelot et al., 2006; Silva et al., 2006) and yoghourt (Korhonen and Pihlanto, 2006) were hailed as having additional benefits to consumers, including the potential to combat cancer.

Enzyme hydrolysis using animal (Lourenco da Costa et al., 2007; Kim et al., 2007b; Saint-Sauveur et al., 2008), plant

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(Peña-Ramos and Xiong, 2001; Kim et al., 2007b), fungal (Rutherfurd-Markwick and Moughan, 2005), and bacterial proteinases (Pihlanto-Leppälä et al., 1999; Lourenco da Costa et al., 2007; Zhong et al., 2007) have increasingly offered better processing conditions due to the mild temperature, pH and minimum sample pre-handling as well as the possibility to dramatically reduce or eliminate wastes. There has recently been a shift from use of the animal to increasing use of microbial proteinases owing to their cost competitiveness and high activity owing to their broad (or lack of) specificity. Nevertheless, the broad spectrum nature of substrate attack coupled with their being less well characterized compared to the animal proteinases means the product composition is generally less controllable, hence unpredictable. Since the value-addedness of the bioactive peptides is a sensitive property which informs the consumer choice, it is becoming increasingly important to generate products whose composition can be anticipated and predicted as accurately as is practically possible.

Besides, since enzyme hydrolysis was heralded as a process which could be controlled, that aspect of control needs to shift from the monitoring of the time, and degree, of hydrolysis and related indices to the actual final product composition. Besides the enzyme and the substrate, hydrolyses processes have generally been carried out with little attention being paid to the holistic influence of the hydrolysis environment on the enzyme, substrate and their interaction (Murthy et al., 1980; De Souza Otero et al., 1980; Baumy and Brule, 1988; Earnest et al., 1991; Diniz and Martin, 1996; Camacho et al., 1998; Iametti et al., 2002; Pelegrine and Gasparetto, 2005; Hiller and Lorenzen, 2008). In order to incorporate more control on the process, it is important that the influence of the hydrolysis environment (pH, temperature and ionic concentration of the hydrolysis buffer) on the hydrolysis product, their sequence of production and composition be elucidated so as to enable the choice of the enzyme be driven by not only the "speed" of hydrolysis, cost of the enzyme and other criteria but the desired product. These have been used as the basis for enzyme choice but in order to give prominence to the key aspect of predictability of enzyme hydrolysis, a key advantage over chemical hydrolysis. In a process which is continually gaining in prominence as a method of choice for value-added product manufacture, more information on what the environment does in driving the enzyme attack sequence, hence the final product composition is becoming increasingly important. This is also driven more by a very knowledgeable and demanding consumer base, what with easy access to information.

Whey proteins

Whey proteins are a by-product of cheese manufacture and potential environmental nuisance (Fig. 1). With advanced technology in its reclamation and purification, however, whey proteins are currently recognized as value-added proteins, and produced as either a concentrate (whey protein concentrate, WPC) or isolate (whey protein isolate, WPI). Whey proteins have, therefore, gained prominence as functional foods used to boost performance diets for athletes owing to their rich supply of the muscle-building lysine amino acids (de Wit, 1998; Cheison and Wang, 2003). In addition, because of their functional properties, they are now applied as emulsifying or foam stabilizers (Dickinson, 2001; Foegeding et al., 2002; Khalloufi et al., 2008; Ye, 2008). Further, it has been found that whey protein functional properties were improved dramatically following hydrolysis (Singh and Dalgleish, 1998; Ipsen et al., 2001) as well as general structuring agents in different food formulations. In addition, whey proteins have gained prominence as fat replacers owing to their low energy properties while offering competitive sensorial properties for which fat is reputed in products like ice-cream, cheese and yoghourts (Lucca and Tepper, 1994; McMahon et al., 1996; Sandoval-Castilla et al., 2004).

Alongside the development of various application products from whey proteins was the emergence of the recognition of the fact that whey protein components possess bioactive and health enhancing properties (Chatterton et al., 2006). Prominently, α-lactalbumin (α-La) was reported to shield against alcohol stress in mice (Matsumoto et al., 2001). In addition, it was recognized that when complexed with some fatty acids like oleic acid, human as well as bovine, α-La became lethal to some cancerous cells (Duringer et al., 2003), a property called HAMLET/BAMLET (human/bovine α-La made lethal to tumour cells) (Svensson et al., 2003; Fischer et al., 2004). Currently, α-La is appreciated as a source of important nitrogen in infant formula, owing to its low allergenic response because it is also present in human milk.

In addition to the acceptance of the whey proteins as both functional and health ingredients, whey proteins are recognized as an important source of a group of peptides which are called bio(logically) active peptides. These peptides possess additional benefits for the consumer apart from supplying the amino acids. They are known to lower blood pressure due to their ability to inhibit the enzyme responsible for high-blood pressure, angiotensin-I converting enzyme (ACE). Other bioactivities associated with whey peptides are antioxidative, antimicrobial, opioid antagonists, anticariogenic, immune-modulating, and ion-binding. Bioactive peptides released from whey proteins were reported to originate from β-lactoglobulin, β-Lg (Hernández-Ledesma et al., 2008), α-La (Kamau et al., 2010), and lactoferrin (Bellamy et al., 1992). Most of the earlier trials were performed using animal proteinases like trypsin, pepsin, and chymotrypsin. Over time, however, many enzymes of microbial, as well as plant origin, gained acceptance owing to their rapid and broad-spectrum hydrolysis potential in addition to their competitive cost. Nevertheless, with this came the possibility to produce even more heterogeneous peptides with little control on their composition because unlike animal proteinases which have narrow substrate specificity, microbial proteinases are less specific and are able to digest many peptide bonds.

This new frontier in the application of protein hydrolysates has attracted many investigations on the potentialities of food proteins being used to deter, delay or cure disease. The peptide mixtures produced in any given enzyme hydrolysis process are too diverse and purification of the bioactive peptide from the mixture was considered a good approach to enrich the bioactivity of hydrolysates. However, with each purification process and step, losses in protein nitrogen with subsequent product cost increases, which mean that the bioactive products were

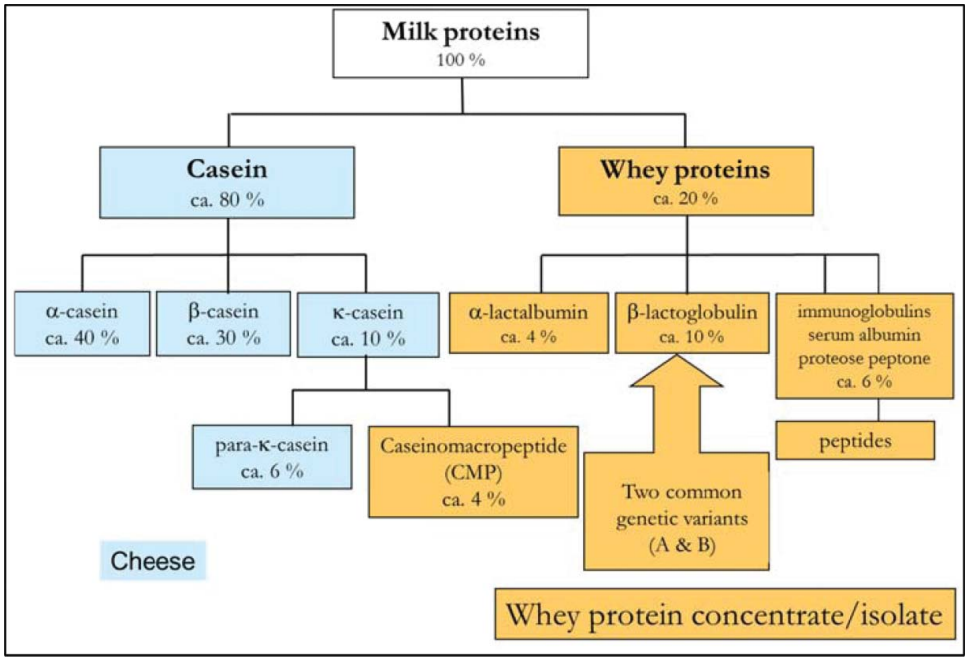

Figure 1. Main components of milk proteins showing partitioning into whey and casein factions. The light blue color codes for cheese proteins while the orange color represent the proteins in whey.

prohibitively high for most consumers. A better approach would be to work predictively in order to control the hydrolysis process and, therefore, tailor the hydrolysis mixtures based on the knowledge of the amino acid sequence of the parent protein and the enzyme hydrolysis specificity.

β-lactoglobulin

The β-lactoglobulin (β-Lg) is a major component of the whey protein fraction in bovine milk, constituting more than 50% of the total whey proteins. It exists in nature at room temperature as a dimer of two noncovalently linked monomeric molecules (McKenzie and Sawyer, 1967). The structure of native β-Lg is well-known from X-ray crystallographic studies and high resolution nuclear magnetic resonance studies as represented in Fig. 2. The secondary structure consists of about 6–10% α-helix, 44–52% β-sheet, 8–10% turn, and 32–35% of random coil (Casal et al., 1988; Dong et al., 1996; Qi et al., 1997). This structure forms nine strands of antiparallel β-sheet and eight of them form a hydrophobic barrel that is bordered by an α-helix on one side (Papiz et al., 1986; Farrell et al., 2004).

Bovine β-Lg has five Cys residues with four of them forming disulphide bridges. The bridges are between Cys^{66} and Cys^{160}, and between Cys^{106} and Cys^{119}. The Cys^{121} is a free thiol group and, therefore, available for reaction between β-Lg and any other protein. The bond of Cys residues Cys^{106} and Cys^{119} as well as Cys^{121} sit within the hydrophobic pocket between one side of the helix and the segments of the G and H strands. The Cys^{106} – Cys^{119} disulphide bond is separated from Cys^{121} by the phenyl ring of Phe^{136} (Creamer et al., 2004a).

The necessary stabilization of the compact globular conformation is also managed by these bonds, whereas one of the disulphide bonds is near the C-terminus (Cys^{66}-Cys^{160}) and one in the interior of the molecule (Cys^{106}-Cys^{119}). The structure has a strong homology with the plasma retinol-binding protein and other proteins that play an important role in physiological pathways. They all share the ability to bind small hydrophobic molecules into a hydrophobic cavity to transport them and so they are all members of the lipocalin family. The exact function of β-Lg is not known yet, but this fact leads to the suggestion that it may act as a transport protein for lipid-soluble biological components such as fatty acids and retinoids (Papiz et al., 1986; Sawyer et al., 1998).

The three-dimensional structure of β-Lg is dependent on different factors like pH, temperature or ionic strength. The most remarkable effect of the pH on these structural changes was reported by Tanford et al. (1959). The bases of their work were observations that a general configurational change

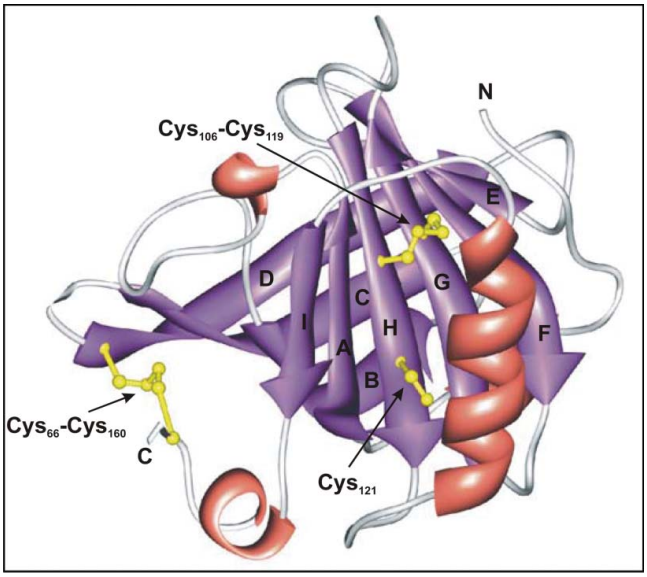

Figure 2. Molecular structure of β-Lg showing N- and C- terminals, the disulphide bridges and free thiol Cys^{121}. Also shown are the α-helix, β-sheet, β-turn, and random coil. Adopted from Tolkach and Kulozik (2007).

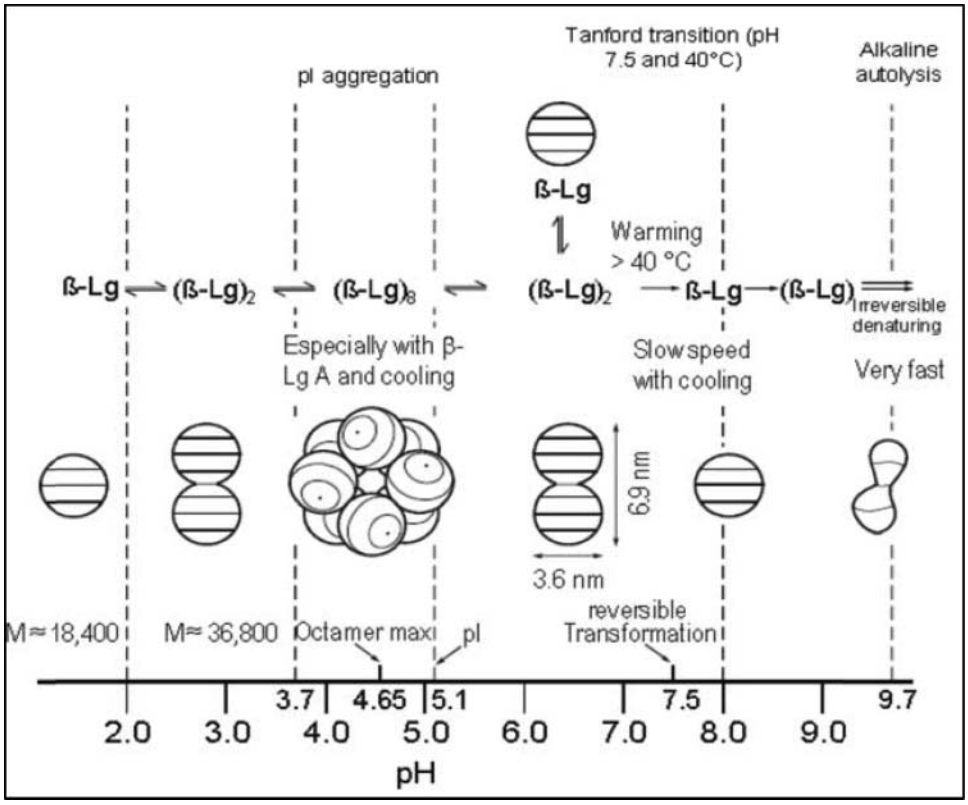

Figure 3. Influence of the temperature and pH on the molecular structure of whey protein β-Lg showing that at pH > 7.51 or temperature above 40°C, the dimers dissociate into monomers. This property influences enzyme interaction with the substrate (adopted from Pessen et al. (1985)).

occurred at the pH near 7.5. They characterized this transition by a release of a buried carboxyl group, an increase in the reactivity of a free sulphydryl group and in a change of the environment of a tyrosine moiety that leads to its exposure. Furthermore, they postulated that the transition that is caused by an increasing pH was not an unfolding like it is the case with other proteins; but was more a kind of new folding. The explanation was that only a part of the protein participates in the reaction and, therefore, β-Lg undergoes a more general slow and irreversible denaturation at pH around 9.5. This indicates that the secondary structure is difficult to alter and once it is altered, it is difficult to regain it. This is confirmed by, for example, the increase of the reactivity of the free sulphydryl group and by the fact that three of the four tyrosine residues start to ionize above pH 9.3, whereas the fourth ionizes only above pH 11 (Tanford et al., 1959; Townend et al., 1969; Casal et al., 1988).

As it can be seen in Fig. 3, the molecular structure of β-Lg strongly depends on the pH. Under physiological conditions of milk (pH around 6.6–6.8), it exists as a dimer. This accumulation of the β-Lg into dimers seems to be very special for ruminants, because in most other species β-Lg appears to be monomeric (Hambling et al., 1992). Decreasing the pH to 3.5 – 5.5, β-Lg A especially forms octamers at temperatures round 4°C, whereas it is assumed, that these are four dimers, most probably with a 422 symmetry (Townend and Timasheff, 1956). These octamers may be stabilized by the presence of carboxyl–carboxylate interactions (Sawyer and James, 1982). By decreasing the pH to between 2 and 3, it tends to dissociate into monomers. Increasing the pH above physiological conditions of pH 6.8 also leads to dissociation of the dimers, but if the pH is raised further it results in a denaturation of the β-Lg. Generally, alkaline denaturation of bovine β-Lg becomes significant above pH 8 and increases with pH increase from 8 to 9. With increasing the pH above 10 further structural changes occur until at pH 12 the structure is completely random (Sawyer, 2003). Therefore, it seems logical that under these autolysis conditions the cleavage is obviously less controllable.

Early work on bovine β-Lg suggested that the dimer dissociation occurs between 30 and 55°C (Sawyer, 1969; Purcell and Susi, 1984), higher temperatures causes unfolding and increased thiol reactivity (Larson and Rolleri, 1955; Sawyer, 2003). The structure of the different genetic variants A and B also influences their behavior under different milieu conditions. The substitution of Asp^{64} to Gly^{64} caused a change of the conformation of the CD loop (Qi et al., 1997) in variant B. The Val^{118} to Ala^{118} exchange resulted in a void, because the bigger isopropyl part of the Val is substituted with the smaller methyl group. Therefore, the B variant is less well packed and this may be responsible for the lower thermal stability of the B variant (Qi et al., 1997).

This genetic effect on the properties of the protein was also investigated by Creamer et al. (2004b). Using SDS-PAGE electrophoresis, they detected the protein concentrations of the different variants A, B, and C after tryptic hydrolysis. The quantity of the bands decreased rapidly over the time for β-Lg A, slowly for β-Lg B and even more slowly for β-Lg C. They also varied the environmental conditions like pH and temperature. It was shown that the rates of hydrolysis increased with

pH for all three variants between pH 7.24 and 8.31. Increasing the pH up to 8.58 and decreasing the E:S ratio from 1:100 to 1:200, the rate of hydrolysis did not change significantly for β-Lg A, whereas the rate decreased for β-Lg B and increased slightly for β-Lg C. Increasing the temperature from 20°C to 57°C led to an increase in the rate of hydrolysis. In our previous work, this genetic-dependent tryptic (Cheison et al., 2011d) and chymotryptic (Lisak et al., 2013) revealed higher susceptibility of genetic variant A than B when hydrolysis was monitored using the sensitive RP-HPLC.

The genetic variant also has a significant effect on the solubility; the native β-Lg B is five times more soluble than β-Lg A (Hambling et al., 1992). A further factor that influences the solubility and the viscosity of the protein structure is the pH. This is a result of the charge of the side groups of the proteins. At high pH, they are deprotonated and at low pH the groups are protonated and, therefore, more soluble. The dimer of native β-Lg is prevalent at room temperature near pH 6.0. The reversible equilibrium $(N_{\beta\text{-Lg}})_2 \leftrightarrow 2\ N_{\beta\text{-Lg}}$ (dimeric-to-monomeric) is driven to the right to form monomers during heat treatment up to 55°C. Between pH 6.5 and 7.8 a reversible conformational change (N ↔ R) from a native (N) to a reversible conformation (R), the Tanford transition (Tanford et al., 1959), is detected by a change in optical rotation. This conformational change of the tertiary structure of monomers results in exposure of the thiol group (Cys^{121}) (Dunnill and Green, 1966), and the exposure and ionization of an anomalous carboxyl group (Glu^{89}), having a pK value of 7.74 during the N ↔ R transition. Qin et al. (1998) observed that the solvent-accessible area of Glu^{89} was pH-dependent. Titration of Cys^{121} with para-mercury benzoate showed that the reactivity of this thiol group increases one order of magnitude within the small pH range $6.75 < pH < 7.05$, and up to four orders at pH 8.5. The Tanford transition, accompanied by the dissociation of dimers, is also thermal induced (at pH > 6.8). According to Qi et al. (1995) the temperature dependent dimer–monomer dissociation takes place only at protein concentrations ≤ 25 mg/mL. During heat treatment the native dimer unfolds directly to a molten globule state at higher β-Lg concentrations. Formation of inter-molecular disulphide bonds is thought to be the major contributory factor in irreversible aggregation of β-Lg, but these are observed at temperatures above 60°C (de Wit, 2009).

α-lactalbumin

Alpha-lactalbumin (α-La) (Fig. 4), a major bovine milk whey protein, consists of 123 amino acid residues forming a compact globular structure stabilized by four disulphide bonds (Cys^6-Cys^{120}, Cys^{61}-Cys^{77}, Cys^{73}-Cys^{91}, and Cys^{28}-Cys^{111}). Under native conditions, the tertiary structure of α-La is composed of a large domain (α-domain) and a small domain (β-domain) divided by a cleft (Pike et al., 1996). The α-domain (134 and 86123) contains three pH-stable α-helices (helix H1, 5-11; H2, 23-34; H3, 8698), a pHdependent α-helix (H4, 105-110), and two short 3_{10} helices (h1, 18-20; h3, 115-118). The flexible 105–110 loop region adopts a helical conformation (H4) at pH between 6.5 and 8.0 (Pike et al., 1996). The 35–85 β-domain is composed of a small three-stranded antiparallel β-pleated sheet (strand S1, 41–44; S2, 47–50; S3, 55–56) and of a short 310 helix (h2, 77-80) (Pike et al., 1996; Chrysina et al., 2000). Calcium

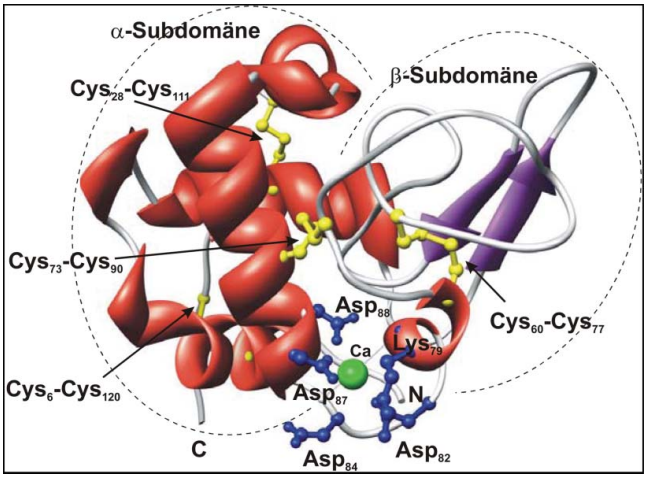

Figure 4. Bovine α-La showing the α- and β-sub-domains and the Cys-Cys disulphide linkages.

binding (association constant of $3 \times 108\ m^{-1}$) at 20°C (Permyakov and Berliner, 2000) strongly influences the molecular stability of α-La and is required for refolding and native disulphide bond formation in the reduced, denatured protein. The calcium-binding loop is located at the junction of the α- and β-domains and partly contains the α-helix H3 and 310 helix h2. The calcium ion is coordinated to β-carboxyl groups of three aspartic acid residues (Asp^{82}, Asp^{87} and Asp^{88}), two backbone carbonyl oxygens ($Lys^{79}$and Asp^{84}), and two water molecules (Acharya et al., 1989; Ravi K. Acharya et al., 1991). Under a variety of conditions (calcium removal, high temperature, strong acid conditions, or presence of denaturing agents), α-La can adopt the "molten globule" conformation, which is described as a compact state keeping the secondary structures, but having a poorly defined tertiary structure. It also has a potential N-linked glycosylated point at Asn^{45} (Ptitsyn, 1995).

Hydrolysis of α-La is difficult to perform, as the compact globular structure is relatively resistant toward enzymatic proteolysis. To increase its susceptibility to proteolysis, the α-La structure has been modified by various conditions. Thus, tryptic hydrolysis at 37°C of α-La is improved by esterification (Sitohy et al., 2001) or by lowering the pH to 2.0 when pepsin is used (El-Zahar et al., 2005). Binding of zinc ions to α-La (zinc to protein ratios up to 20) also increases the protein susceptibility to enzyme digestion (Permyakov et al., 1991). The presence of 50% trifluoroethanol induces conformational change leading to limited proteolysis of α-La by thermolysin at room temperature (Polverino de Laureto et al., 1995). α-La is considered to be the most heat-stable whey protein. Heat treatment of α-La prior to enzyme hydrolysis does not improve the proteolysis significantly, as the temperatures used [usually less than the temperature of irreversible denaturation of 100°C (Boye et al., 1997)] allow the protein to refold during the cooling stage (Schmidt and Poll, 1991; Schmidt and van Markwijk, 1993).

State of knowledge in protein hydrolysis

Protein hydrolysis

Protein hydrolysis involves, quite simply, the addition of a split water molecule to the peptide bond resulting in its partitioning

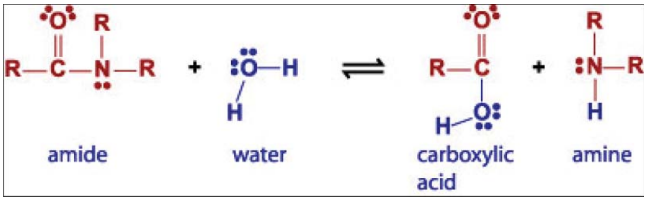

Figure 5. Theory of protein hydrolysis mechanism in which a molecule of water is added to split the amide bond.

to release the two termini with a carboxylic acid group (C-terminal) and an amino group (N-terminal) (Fig. 5). Enzymes are generally specific to the cleavage of the amide (N-C) bond formed. Some proteinases are more specific to particular bonds formed by specific amino acids, or between particular amino acids and any other amino acid. An enzyme like bovine trypsin (EC 3.4.21.4) is reportedly specific to the cleavage of peptide bonds formed between any other amino acid on the C-terminals of either a Lys or Arg, except where that other amino acid is a Pro. An enzyme like LysC is narrowly specific to cleavage points similar to those of trypsin except it doesn't cleave at arginyl residues.

Consequent to the cleavage of peptide bonds, the terminals are variably ionized relative to certain conditions of hydrolysis, mainly pH and temperature. Hence, during hydrolysis at acid pH, there is release of OH^- groups, whereas hydrolysis in alkaline region results in release of H^+ (Fig. 6). Consequently, at acid pH hydrolysis, the pH keeps increasing during hydrolysis while during hydrolysis at alkaline pH there is a concomitant drop in the pH. Because of the buffering effect of proteins, particularly milk proteins, it is likely that the change in pH is not entirely proportional to the release of the hydrogen and/or hydroxyl ions. Because of the sensitivity of enzymes to the pH due to the likelihood to denature at pH values away from the optimum pH, hydrolysis pH is regulated using pH adjusting buffers or, where that can be avoided, simply with NaOH and/or HCl of concentrations not exceeding 2 mol/L in order not to denature the protein. Since there is a good correlation between the amount of pH-changing species and the extent of hydrolysis, there is a well-established link between that amount and the degree of hydrolysis (DH) to which the proteins are hydrolyzed (Camacho et al., 2001). This quantity (DH) is an important index of protein

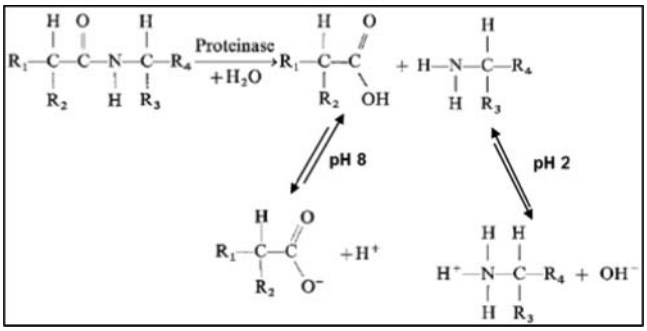

Figure 6. Peptide hydrolysis showing ionization possibilities at pH 2 and pH 8 resulting in pH changing OH^- and H^+ ion release.

hydrolysis and determines several properties of the hydrolysates (Adler-Nissen, 1986).

By definition, the DH is given by Equation (2.1):

$$DH = \frac{h}{h_{tot}} \tag{2.1}$$

Where:

h = number of peptide bonds hydrolyzed
h_{tot} = maximum number of peptide bonds in a protein (given in mequivalents/g protein)

Since the pH-correcting solution correlates linearly with the DH, it is possible to modify the relationship in Equation (2.1) so that the DH can be calculated using the pH-stat(ic) method using the relationship in Equation (2.2) in the case of hydrolysis under alkaline conditions (Adler-Nissen, 1986):

$$DH = V_{alkali} * N_{alkali} * \frac{1}{M_{protein}} * \frac{1}{h_{tot}} * \frac{1}{\alpha} * 100 \tag{2.2}$$

Where:

DH = degree of hydrolysis
V_{alkali} = volume of alkali consumed to control the pH during hydrolysis
N_{alkali} = normality of alkali consumed to control the pH during hydrolysis
$\frac{1}{M_{protein}}$ = mass of % nitrogen as protein in sample
$\frac{1}{h_{tot}}$ = total hydrolysable peptide bonds in protein in meqv/g
$\frac{1}{\alpha}$ = degree of ionization of the α-amino group in a protein sample

When hydrolysis is performed under acid pH, the volume of HCl used to regulate the pH is also proportional to the DH. However, the specific group of interest is the carboxylic acid group and the ionization of the OH^- ions whose liberation leads to an increase in the pH. In that regard, a modification of Equation (2.2) according to Diermayr & Dehne (1990) gives Equation (2.3) which is useful in calculating the DH using the pH-stat method in acid region hydrolysis.

$$DH = \frac{F_{pH} * V_{HCl} * N_{HCl}}{M_P * h_{tot}} * 100 \tag{2.3}$$

Where:

$F_{pH} = \frac{1}{1-\alpha}$ = is a correcting factor and is influenced by the pH value between 25°C and 50°C. The value depends on the dissociation constant of the carboxylic acid group (COOH)
DH = degree of hydrolysis
V_{HCl} = volume of HCl consumed during hydrolysis
N_{HCl} = Normality of HCl used to maintain the pH at constant experimental value

M_p = mass of protein in the substrate (% nitrogen content)
h_{tot} = total number of available peptide bonds
α = the dissociations degree of the COOH group defined by Equation (2.4)

$$\alpha = \frac{10^{pH-pK_A}}{1+10^{pH-pK_A}} \quad (2.4)$$

The DH provides a rough estimate of the peptide sizes in the hydrolysates, the so-called peptide chain length (PCL), which is mathematically the inverse of the DH (Adler-Nissen, 1986). Moreover, there is a close correlation between the time of hydrolysis as measured by the DH and hydrolysate properties such as allergenicity, functional properties, and bioactivity (Mahmoud et al., 1992; van der Ven et al., 2001; van der Ven et al., 2002a; van der Ven et al., 2002b) although it was not found to affect antioxidant activities of whey protein hydrolysates (Peña-Ramos and Xiong, 2001). In addition, the DH affects peptide bitterness, a property which is likely to negatively influence the applicability of protein hydrolysates (Cheison et al., 2007c). Nevertheless, the DH is an oft used protein hydrolysis index other than the time of hydrolysis because of the ease with which the hydrolysate sizes can be related to it. The pH-stat is the easiest to compute and is a more straightforward index of protein susceptibility to enzyme hydrolysis.

Enzyme reaction in detail

The enzyme hydrolysis process proceeds in an environment that provides the interaction and conversion conditions between the substrate-enzyme complex and the product-enzyme on the other end of the reaction scheme. A good understanding of the hydrolysis process and the forces involved would help elucidate the role of the milieu in the way the environment influences the enzyme attack patterns and product composition. In trypsin, the process begins with the backbone of the substrate peptide binding adjacent to the catalytic triad then the specific side chain fits into its pocket. Asp^{102} of the catalytic triad positions His^{57} and immobilizes the substrate through a hydrogen bond as shown (Fig. 7) (Parekh et al., 2011). In the first step of the reaction, His^{57} acts as a general base to withdraw a proton from Ser^{195}, facilitating a nucleophilic attack by Ser^{195} on the carbonyl carbon of the peptide bond to be cleaved. This is probably a concerted step, because proton transfer prior to Ser^{195} attack on the acyl carbon would leave a relatively unstable negative charge on the serine oxygen.

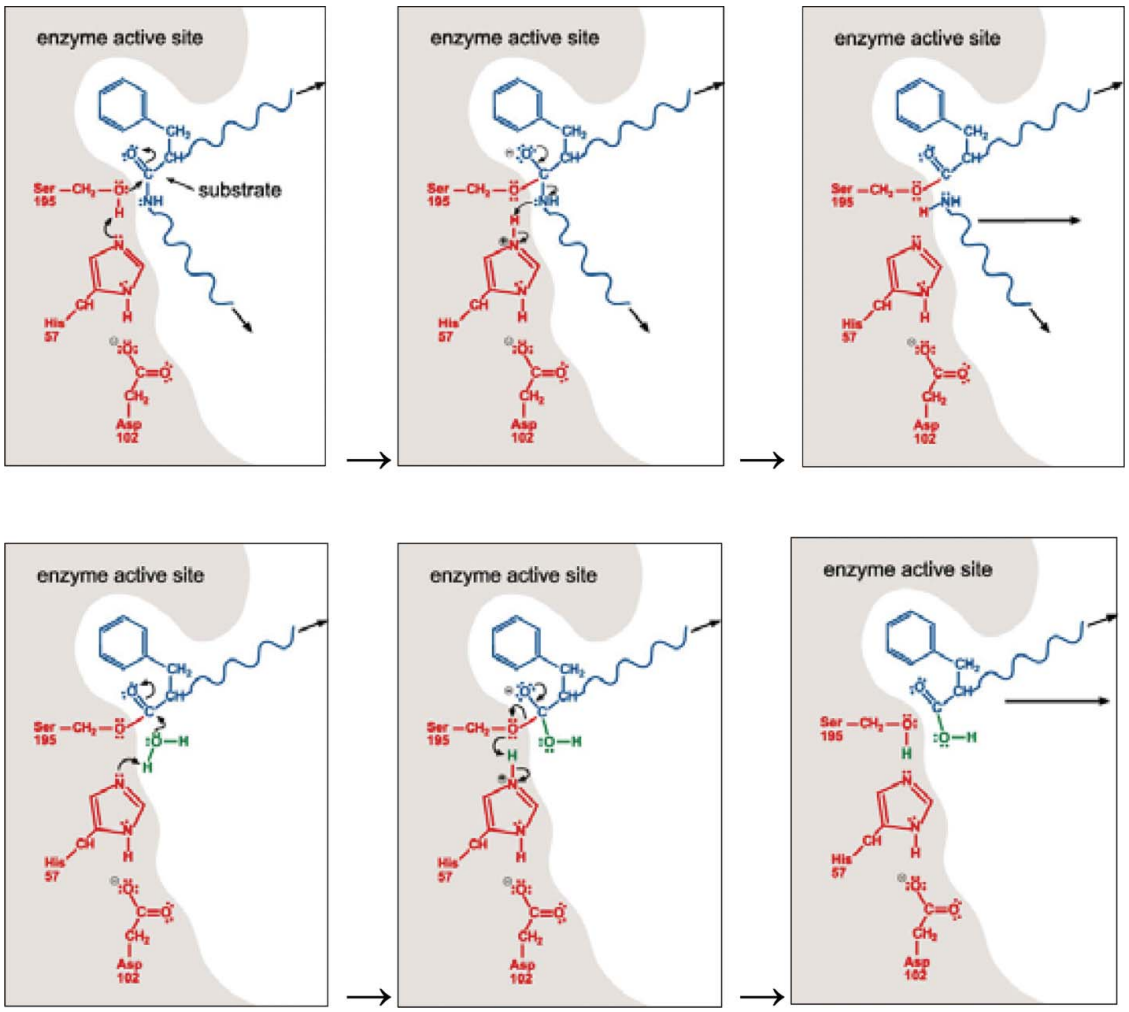

Figure 7. Mechanism of substrate attack in serine proteases in which the process begins with His^{57} donating a proton to the Ser^{195} (Source Parekh et al. (2011)).

In the next step, donation of a proton from His^{57} to the peptide's amide nitrogen creates a protonated amine on the covalent, tetrahedral intermediate, facilitating the subsequent bond breaking and dissociation of the amine product. The negative charge on the peptide oxygen is unstable; the tetrahedral intermediate is short-lived and rapidly breaks down to expel the amine product. The acyl-enzyme intermediate that results is reasonably stable; it can even be isolated using substrate analogues for which further reaction cannot occur. With normal peptide substrates, however, subsequent nucleophilic attack at the carbonyl carbon by water generates another transient tetrahedral intermediate. His^{57} acts as a general base in this step, accepting a proton from the attacking water molecule. The subsequent collapse of the tetrahedral intermediate is assisted by proton donation from His^{57} to the serine oxygen in a concerted manner. Deprotonation of the carboxyl group and its departure from the active site completes the reaction as shown (Fig. 7). The enzyme, upon release of the product, is ready for subsequent conversion. This process takes place is milliseconds.

The aforementioned events indicate the role of water molecules both in the hydrolysis reaction as well as providing the enabling environment in which proton exchange takes place. There seems to have been little attention paid to the effect of the hydrolysis environment on the protein structure, enzyme stability and the interaction between the substrate and the enzyme and hence its effect on enzyme specificity and hydrolysate composition. Most reports rely on the declared enzyme hydrolysis properties and activity at optimal conditions while ignoring the contributing factors which might impact on both the enzyme and substrate properties like solubility, viscosity, gelation, charge properties and stability. Furthermore, since most studies are confined to the hydrolysis processes at enzyme declared optimal conditions, little is known about the hydrolysis properties of an enzyme below or above those optimal conditions. The main decision factor in choosing an enzyme seems to be the speed of hydrolysis rather than the final hydrolysate composition and whether that composition can be influenced by choice of different conditions other than the optimal combinations. It would be interesting to determine the nature of hydrolysis processes that proceed at sub-optimal conditions while working in regions of possible suppressed enzyme activity so as to improve the possibility to control enzyme attack.

The enzyme trypsin

Trypsin (EC 3.4.21.4), a serine protease of the S1 family, belongs to a class of proteolytic enzymes whose active-site is a serine residue. It has a pI of between 10.1 to 10.5 and molecular weight of 23.3 kDa (Walsh, 1970). Its pH stability range is 7 to 9 and is activated and stabilized by Ca^{2+} ions (Sipos and Merkel, 1970). In this group of serine proteinases are to be found chymotrypsin, elastase, thrombin, subtilisin, and plasmin.

Trypsin is synthesized in the pancreas and secreted into the digestive tract as inactive proenzyme, or zymogen (trypsinogen in the case of trypsin) (Fig. 8). Bovine trypsinogen consists of a single polypeptide chain of 229 amino acids and is cross-linked by six disulphide bridges. Within the digestive tract enterokinase, or autolytic activity by already activated trypsin, activates pancreatic trypsinogen to trypsin by the hydrolysis of a signal hexapeptide (for bovine trypsin at the Lys^6-Ile^7 peptide bond) from the NH_2 terminus (Fig. 8). The enzyme trypsin, therefore, consists of a single chain polypeptide of 223 amino acid

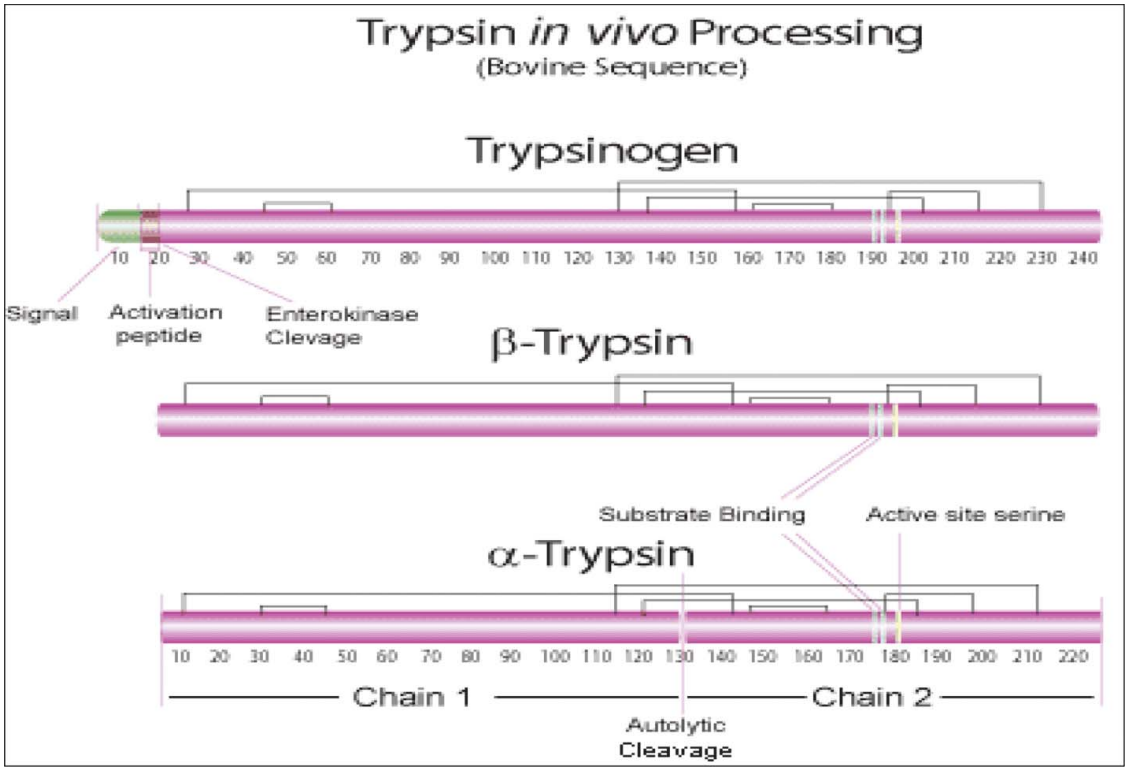

Figure 8. Trypsinogen and its activation to trypsin showing additional cleavage sites to plroduce α-trypsin, β-trypsin, and cationic trypsin.

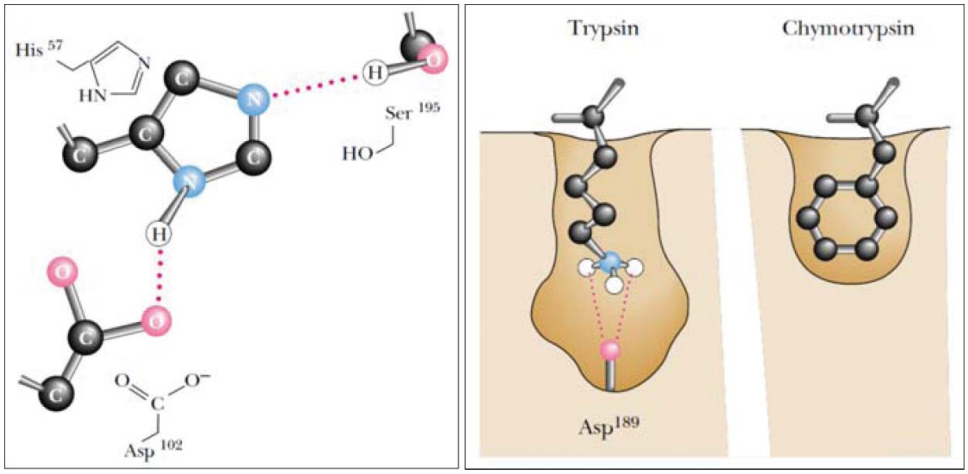

Figure 9. The serine protease catalytic triad and (left) and the catalytic pockets (right) in trypsin (deep narrow) compared to that in chymotrypsin (shallow and wide).

residues. This native form of trypsin is referred to as β-trypsin. Autolysis of β-trypsin (which is cleaved at Lys^{131}-Ser^{132} in the bovine sequence) results in α-trypsin which is held together by disulphide bridges (Fig. 9). Further degradation of a-trypsin leads to pseudotrypsin (ψ-trypsin) in which an additional bond is opened, between Lys^{176} and Asp^{177}. This structural change occurs at the direct vicinity of Asp^{177}, which represents the specificity site of trypsin for the binding of positively charged substrates (Keil-Dlouhá et al., 1971a).

Pseudo-trypsin is produced over long storage of trypsin and its activity resembles that of chymotrypsin (Keil-Dlouhá et al., 1971a) although it showed low affinity for polypeptide substrates. Trypsin shares about 40% amino acid homology with chymotrypsin as well as the conserved catalytic triad (Fig. 9). This property has been claimed to explain the apparent loss of the narrow trypsin in studies that showed that trypsin catalyzed chymotryptic-specific peptide bonds like Ty-Ser (Asao et al., 1992).

During hydrolysis of proteins, trypsin undergoes self-hydrolysis, or autolysis, to release several peptides which are sometimes mistaken for protein fragments in hydrolysate analysis (Harris et al., 1990). This is because owing to its specificity for hydrolysis of amide bonds formed by lysyl and arginyl residues (Fig. 10), trypsin has, within its sequence, 14 lysyl and two arginyl residues which are potential attack sites for self-hydrolysis (Vestling et al., 1990). A departure from that narrow specificity was detected with trypsin hydrolyzing both the N-terminal of lysine residues and specifically at the C-terminals of asparagine, and tyrosine, a property which has been reported by many previous investigators working with high purity, L-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Greene and Giordano, 1969; Asao et al., 1992; Maynard et al., 1998).

Trypsin is the most commonly used enzyme for specific proteolysis in proteomics because of its narrow specificity. The number of smaller peptides resulting from trypsin action is equal to the total number of Arg and Lys residues in the protein plus one, the protein's C-terminal peptide fragment. According to the nomenclature of Schechter and Berger (1967), the lysine or arginine residue involved in the scissile bond is defined as the P_1 residue. The residues extending towards the amino-terminal side of the protein are P_2 to P_n and those extending towards the carboxy-terminal side of the protein are noted P_1' to P_n'. Tryptic activity is modulated by five or six residues

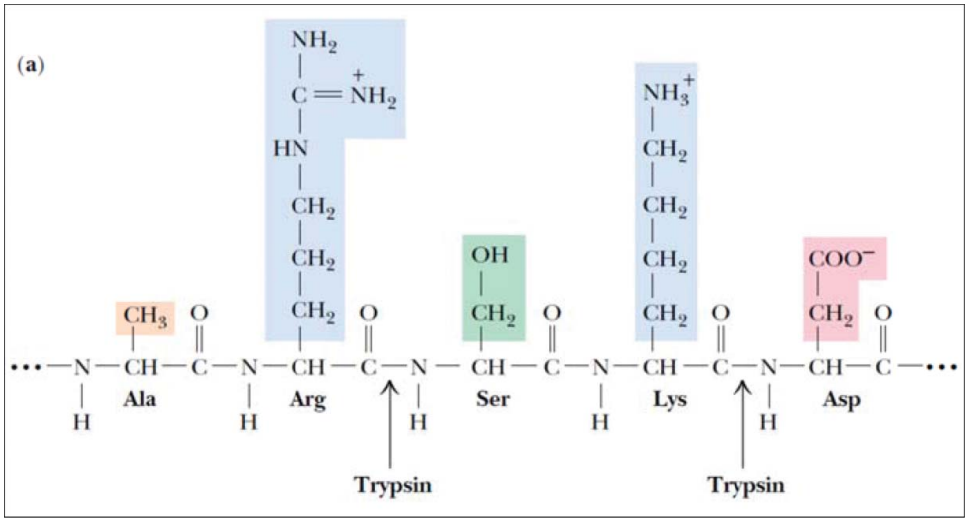

Figure 10. Schematic representation of trypsin hydrolytic attack sites in a protein peptide matrix showing Lys and Arg residues (source Garrett and Grisham (2007)).

surrounding the cleavage site (from P_3 to P_3'). These residues, except the P_1 residue, define the secondary specificity as demonstrated using synthetic substrates (Schellenberger et al., 1993; Schellenberger et al., 1994). Aliphatic residues are preferred in P_1' and P_2' positions followed by aromatic and basic residues whereas Pro and acidic side chains are particularly disadvantageous. The influence of the residue diminishes from P_1' to P_3' position.

Little data is available on the influence of P_2 to P_4 residues; nevertheless, a carboxylic group in P_2 position decreases the rate of hydrolysis (Mihalyi, 1972). The accessibility of the scissile bond towards the enzyme is a very important factor for hydrolysis. The rate of hydrolysis is slower if an acidic residue is on either side of the cleavage site and no cleavage occurs if a proline residue is on the carboxyl side of the cleavage site. Indeed it was reported that the amino acids at up to the tenth position on either side of the scissile bond influence the susceptibility of substrates (Schellenberger et al., 1993).

Trypsin has three polar residues namely His^{57}, Asp^{102}, and Ser^{195}, which form the catalytic triad at the active site (Fig. 9). This triad is also conserved in chymotrypsin. This conservation of the catalytic triad, as well as the conservation of the amino acid sequence has been proposed to account for the apparent switch between tryptic and chymotryptic specificity during hydrolysis with trypsin. The two enzymes, both members of the S1 superfamily, probably shared a primitive gene in their evolution. A closer examination of the enzymolysis process reveals that substrate binding and conversion play a central role. This review will not delve into the debate regarding which theories best explains hydrolysis of substrates with regard to the lock-and-key and/or induced fit hypothesis (Garrett and Grisham, 2007).

The hydrophobic walls of the catalytic pocket create a favorable environment for the long aliphatic and unbranched parts of the basic arginine and lysine. The optimal temperature and pH for the performance of trypsin are 37°C and pH 7.8 (Olsen et al., 2004). At 55°C, it was observed that trypsin activity decreased quickly (Galvão et al., 2001). At low temperature, its denaturing is slow. Between pH 6 and pH 4.25, trypsin is also slowly denatured but below pH 4.25 it rapidly loses its activity (Murthy et al., 1980).

Deviations from trypsin-specificity have been reported, however, where it acquired chymotrypsin-like activity which was attributed to a "chymotrypsin memory" (Cheison et al., 2010) in its activity owing to the high sequence homology with chymotrypsin and production of pseudotrypsin with long storage (Keil-Dlouhá et al., 1971a). For example, the peptide bond Tyr-Ser was reported to be scissile to trypsin in earlier works on different substrates (Greene and Giordano, 1969; Asao et al., 1992) including during hydrolysis under pressure (Maynard et al., 1998) which were attributed to trypsin and not contaminating enzymes. Similar trends were observed with the hydrolysis of glucagon where crystalline trypsin also cleaved the carboxy terminals of phenylalanine and tryptophan (Keil-Dlouhá et al., 1971b). In our work, it was observed that trypsin shifted to the chymotrypsin-like hydrolysis pattern as a result of the changes in the temperature and pH of hydrolysis (Cheison et al., 2010).

Hydrolysis of whey proteins

Protein hydrolysis leads to the improvement of the functional and nutritional properties without deterioration of the nutritional value. Hydrolysis of proteins has been achieved using acids, alkali, and enzymes (Fox et al., 1982) as well as supercritical water (Espinoza et al., 2012). Hydrolysis of WPI is performed to improve heat stability (Fox and Hearn, 1978; Shimoyamada et al., 1996), reduce allergenicity (Mahmoud et al., 1992), improve digestibility (Potier and Tom, 2008), and liberate bioactive peptides (Korhonen and Pihlanto, 2006). Enzymes are preferable because they are generally accepted as safe (GRAS) while hydrolysates are generally produced under mild pH and temperature conditions which eliminates the formation of side-reaction products which were observed at elevated temperatures and pH, such as the foulant lysinoalanine. At the same time, because enzyme hydrolysis is a property of both the enzyme and the protein, enzyme specificity and amino acid sequences in the proteins means that enzyme hydrolysis leads generally to the production of less free amino acids unlike in the case of chemical hydrolysis. Some amino acids like tryptophan, asparagine and glutamine are lost during chemical hydrolysis which in the case of asparagine and glutamine is due to their conversion to the acid derivatives aspartic and glutamic acid, respectively (Whitaker, 1977).

Many enzymes have been used to hydrolyze whey proteins. They include enzymes from animal sources like freely trypsin (Chobert et al., 1988; Pouliot et al., 2000), chymotrypsin (Galvão et al., 2001), and pepsin (Mota et al., 2004). In addition, plant enzyme papain (Schmidt and van Markwijk, 1993), Cardosin A (Barros et al., 2003) as well as extracts from *Cynara cardunculus* (Barros and Malcata, 2002) were used. The emergence of microbial enzymes created a dramatic shift to their use because microbial enzymes possess broad selectivity and hydrolyzed proteins to higher DH values. Thus whey proteins were hydrolyzed using alcalase (Doucet et al., 2003), Debitrase (Spellman et al., 2003), flavorzyme (Cheison et al., 2007a), neutrase (Mutilangi et al., 1996), Protease N (Cheison et al., 2007b; Cheison et al., 2007c), Protease A (Cheison et al., 2007a; Martínez-Araiza et al., 2012), and protamax (Schober et al., 2012).

Colostrum whey, containing bovine serum albumin (BSA), α-La and β-Lg was hydrolyzed using a number of enzymes. Results showed that significant amounts of BSA, β-Lg, and α-La survived papain digestion. In contrast, pepsin completely removed BSA but not β-Lg present in heated colostral whey. Alcalase completely eliminated BSA, β-Lg and α-La in colostrum (Kim et al., 2010). Besides, heated and native whey were hydrolyzed using pepsin and trypsin (Kim et al., 2007a). The α-La in native WPC was slightly degraded when incubated with 0.1% pepsin and then with 0.1% trypsin while being almost completely hydrolyzed within 60 min of incubation with 0.5% pepsin and then with 0.5% trypsin. Incubation of native WPC with 1% pepsin and then with 1% trypsin for 30 min completely removed the BSA and α-La. The β-Lg in native WPC was not affected by the pepsin and trypsin. In contrast, β-Lg in heated WPC was partially hydrolyzed by the 0.1 and 0.5% pepsin and trypsin treatments and was completely degraded by the 1% pepsin and trypsin treatment. Antigenicity reversibly mimicked the hydrolysis of WPC and the removal of β-Lg from hydrolysates.

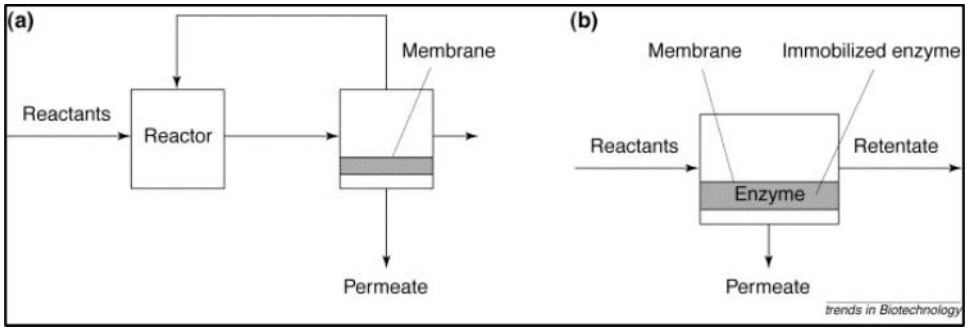

Figure 11. Main configuration types of membrane reactors: (a) a reactor combined with a membrane operation unit, (b) a reactor with the membrane active as a catalytic and separation unit (source Giorno and Drioli (2000)).

The microbial proteases generally attack random peptide bonds, leading to extensively hydrolyzed products which are objectionably bitter, a property which was attributed to high DH and the presence of short hydrophobic peptides (Spellman et al., 2005; Cheison et al., 2007c; Leksrisompong et al., 2010). Because the objective of hydrolysis is to obtain a product with favorable functional and bioactive properties, it would be important to ensure that the sensory properties are not objectionable. Bitter hydrolysates have been debittered using a number of methods which include further hydrolysis using an exopeptidase, plastein reaction to increase peptide length, selective extraction of bitter peptides using activated carbon and macroporous resins (Lalasidis and Sjoberg, 1978; Saha and Hayashi, 2001; Cho et al., 2004; Bruce and Pawlett, 1997; Cheison et al., 2007c). Although the DH can be controlled so as not to proceed extensively, and the type of enzyme used to produce hydrolysates at high DH did not show any significant difference (Slattery and FitzGerald, 1998).

It was also claimed that the bitterness of the hydrolysates was linked to the peptide structure, with terminal amino acids accounting for some prominent bitter tastes apart from the presence of hydrophobic amino acids (Ishibashi et al., 1988; Kukman et al., 1995). Bulky hydrophobic amino acids at the C terminus and bulky basic amino acids at the N terminus were highly correlated to bitterness (Kim and Li-Chan, 2006). This property of peptides is amenable to the enzyme used and the amino acid sequence of the protein because each enzyme has a unique hydrolytic pattern which means the terminal amino acids would depend on how the protein is attacked by an enzyme. Therefore, by choice of the enzyme, peptide bitterness may also be influenced.

Protein hydrolysis using immobilized enzymes. Whey protein hydrolysis was performed using enzymes in free solution, immobilized enzymes (Barros et al., 2003; Marques et al., 2011; Rocha et al., 2011) or in enzymatic membrane reactors (Perea and Ugalde, 1996; Guadix et al., 2006; Cheison et al., 2006a; Cheison et al., 2006b; Prieto et al., 2007) (Fig. 11). Membranes may be of microfiltration (MF), ultrafiltration (UF), or nano-filtration (NF) sizes and they may be used as separation units (Fig. 11(a)) or as active enzyme entrapments (Fig. 11(b)). Where enzymes are immobilized within or on the membrane, the configurations come in different forms as summarized in Fig. 12.

The use of immoblized enzymes and membrane reactors provide advantages like the production of enzyme-free

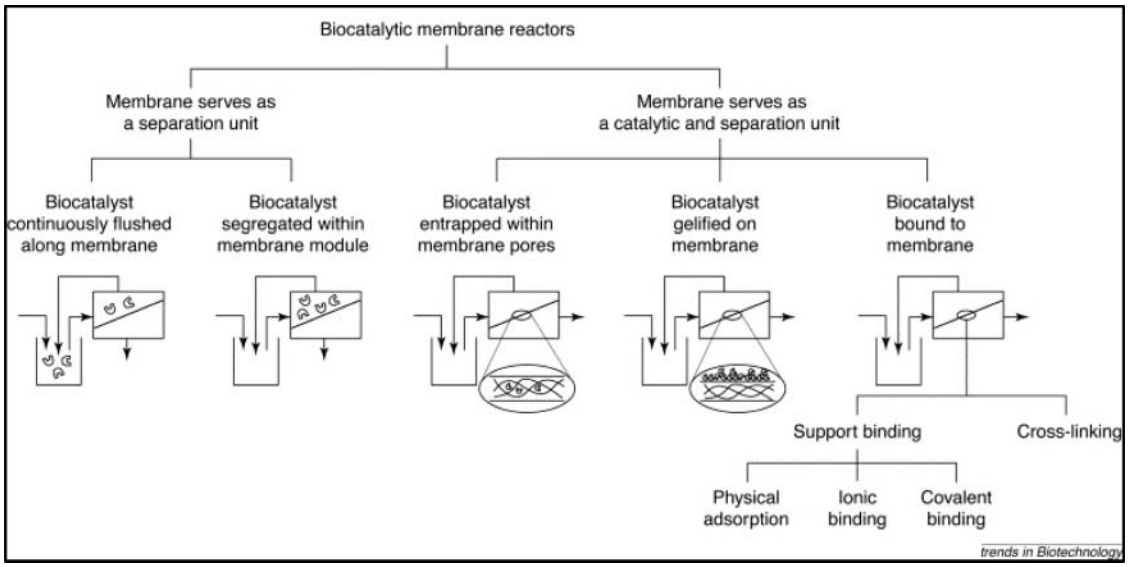

Figure 12. Examples of biocatalytic membrane reactors with enzymes immobilized using different methods (source Giorno and Drioli (2000)).

hydrolysates, obviation of enzyme denaturing at the end of the hydrolysis, enzyme re-use cuts down on the costs while the product removal favors enzyme hydrolysis kinetics due to the elimination of product inhibition. It still remains a challenge, however, due to leakage of the enzyme which leads to limited contamination of the product (Cheison et al., 2006b).

Although membranes hold potential, challenges with regard to their operational stability due to flux declines were reported. Nevertheless, whey protein hydrolysates with specific properties like low immunogenicity and antigenicity (Guadix et al., 2006; Prieto et al., 2007; Cheison et al., 2007b), improved interfacial and emulsifying activity (Turgeon et al., 1991) as well as bioactive peptides (Bordenave et al., 1999; Gauthier and Pouliot, 2003) have also been produced in EMRs.

Protein hydrolysis under high pressure. In addition, high-pressure (HP) treatment on the hydrolysis of dairy whey proteins by trypsin, chymotrypsin and pepsin was analyzed (Peñas et al., 2006). Isostatic pressure (100–300 MPa for 15 min at 37°C) was applied to the protein substrate prior to its enzymatic hydrolysis. Digestion was also conducted at atmospheric pressure (0.1 MPa) and under high pressure. Results suggest that HP increased the DH reached under each of the enzyme. Chymotrypsin and trypsin showed the highest proteolysis at 100 and 200 MPa followed by pepsin at 300 MPa. The β-Lg was hydrolyzed by trypsin and chymotrypsin both pressures, whereas the pepsin only hydrolyzed this protein under HP. In contrast, pepsin and trypsin hydrolyzed α-La in all cases while it remained resistant to chymotrypsin irrespective of the pressure applied.

Beran et al. (2009) studied the effect of isostatic 500 MPa pressure on tryptic and chymotryptic hydrolysis of α- and β-casein, BSA, β-Lg and α-La. Digestion was also conducted at atmospheric pressure. The extent of hydrolysis and peptide profiles was analyzed by gel-permeation and reversed-phase high-performance liquid chromatographies (RP-HPLC). The residual immunochemical reactivities of the protein hydrolysates were assessed by the Streptavidin ImmunoCAP system (Phadia) for determination of specific immunoglobulin type E (IgE) antibodies. Significant changes of the peptide profiles and a progressive reduction in residual-intact proteins were found after applying high pressure during tryptic proteolysis of β-Lg and BSA and chymotryptic proteolysis of β-Lg, α-La, and BSA. A statistically significant decrease of the residual immunochemical reactivities of β-Lg tryptic and α-La chymotryptic hydrolysates prepared under HP, in comparison with the control samples hydrolyzed at atmospheric pressure, was also observed.

Nakamura et al. (1993) compared whey protein denaturation by HP and heat and found that β-Lg and not α-La HPLC profiles changed under HP treatment. Furthermore, HP-treated WP was hydrolyzed with papain and proleather. HPLC profiles of hydrolysates with HP treatment were similar to those of hydrolysates obtained after heat treatment while the antigenicity of hydrolysates treated at 600 MPa without holding were similar to those of hydrolysates treated at 60°C for 30 min. The researchers suggested that HP was superior to heat treatment in the case of hydrolysis of heat-stable protein.

In a series of studies on the HP effect on WP hydrolysis, Chicon et al. (2008) observed formation of protein aggregates when WPI and β-Lg treated at 200 and 400 MPa at pH 6.8. Treatment of β-Lg at 400 MPa increased hydrolysis by pepsin, an advantage which was lost progressively during the refrigerated storage of the HP-treated WPI. Furthermore, β-Lg dissolved in buffer with chymotrypsin and trypsin showed improved susceptibility to both enzymes (Chicon et al., 2008). HP treatment showed that β-Lg hydrolysis by chymotrypsin was similar to trypsin hydrolysis while it proceeded progressively at both atmospheric and HP (Chicon et al., 2006b). It was postulated that HP promoted dimer-monomer transition in β-Lg, hence the improved hydrolysis noted.

Trypsin hydrolysis β-Lg

Whey protein β-Lg has been hydrolyzed using many enzymes. Otte et al. (1997) for example hydrolyzed it using bromelain, papain, pepsin, trypsin, endoproteinase Arg-C, aminopeptidase, and carboxypeptidase Y. Results showed that both papain and trypsin degraded β-Lg with the formation of medium-sized peptides (1–5 kDa), of which seven were identified. This finding means that no two enzymes likely hydrolyze the same protein in a similar pattern. Trypsin and leucine aminopeptidase M (LAP) were also used to digest a number of proteins including β-Lg (Doucette and Li, 2001). Enzyme showed different attack patterns which was visible in the resulting peptide sizes. Differences in the susceptibility of trypsin of β-Lg genetic variants A, B, and C were demonstrated by Creamer et al. (Creamer et al., 2004b) while human trypsin hydrolyzed caseins faster than both α-La and β-Lg (Jakobsson et al., 1983).

With the knowledge of the amino acid sequence of whey proteins, it is now possible to predict the likely composition of peptides issuing from enzyme hydrolysis especially if the enzyme used has well-characterized specificity. With an enzyme like trypsin whose likely attack sites are known, it is easy to predict the attack sites (Fig. 13) in the protein and therefore generate a theoretical library of peptides. In the case of trypsin hydrolysis of β-Lg, for example, there are 15 lysyl and three arginyl residues, which means there are 18 potential trypsin scissile peptide bonds. Of note, one lysyl residue is linked to a proline amino acid, which makes it potentially resistant to trypsin as observed by Olsen et al. (Olsen et al., 2004) who reported that trypsin was exclusively selective to lysine-X and arginine-X peptide bonds in which X was any other amino acid except a proline. Theoretically, therefore, the maximum number of peptides that can be obtained from a total trypsin hydrolysis of β-Lg is given by Equation (2.5):

$$\mathrm{Pep}_{\max} = \sum_{i=1}^{n+1} n_i \tag{2.5}$$

Where:

Pep_{max} = maximum number of peptides with trypsin hydrolysis

$n = 18$ for trypsin hydrolysis of β-Lg

This scenario results in the release, for example between $\mathrm{Pep}_{\min} = 19$ and $\mathrm{Pep}_{\max} = 190$ possible peptides during 100% trypsin hydrolysis of β-Lg. This does not exclude some trypsin-

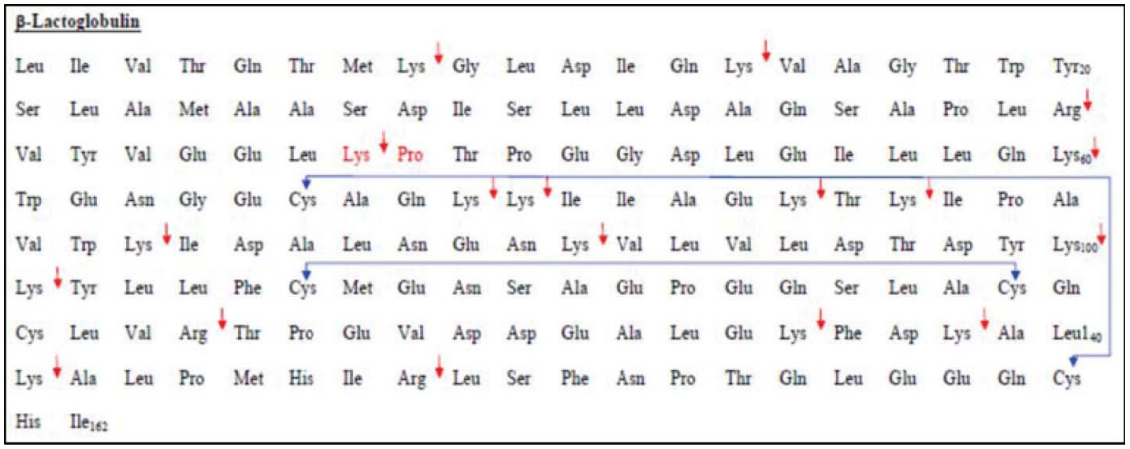

Figure 13. Amino acid sequence of bovine β-Lg showing the potential trypsin attack sites (lysyl and arginyl residues) highlighted, as well as the potentially trypsin-resistant Lys^{47}-Pro^{48} bond. Intra-molecular cross-links are also shown.

resistant β-Lg, which would remain intact under hydrolysis conditions. In addition, there are likely to be tryptic peptides originating from autolysis of the enzyme. The products of tryptic digest of β-Lg would range from single amino acid Lys^{70} to large polypeptides. Some peptides may be cross-linked because of the presence of disulphide bridges which are not severed by the enzyme, and it is important that while scouting for the hydrolysis products such products are considered (Cheison et al., 2010).

Substrate amino acid sequence and predicted hydrolysate composition only predicts where the enzyme is likely to attack. In the case of trypsin, it is already known that arginyl residues are up to 25-fold more easily hydrolyzed than are lysyl residues (Wang and Carpenter, 1967). Arginine has higher pI than lysine and hence potential for higher positive charge at low pH, although the neighboring amino acids play a significant role in influencing trypsinolysis (Schellenberger et al., 1993). This rapid hydrolysis of arginyl residues was however lost at low enzyme-to-substrate ratios during tryptic hydrolysis of B-chain of insulin by Bowman (1979). This interesting finding introduces the question about the sequence of attack of trypsin scissile peptide bonds during trypsin hydrolysis.

For example, with substrate β-Lg, which peptide bonds are attacked initially by the enzyme and which ones are attacked last? In membrane reactor, for example, Martin-Orue et al. (1999) reported that hydrolysis of κ-casein fragment f(106–169), called caseinomacropeptide (CMP) proceeded in a manner that the hydrolysis of the three susceptible bonds, Lys^{111}–Lys^{112}, Lys^{112}–Asn^{113} and Lys^{116}–Thr^{117}, favored the first two. Hence, a kinetic study in the batch system showed that the overall catalytic process followed a sequential mechanism where the Lys^{116}–Thr^{117} bond was only cleaved after the cleavage of the Lys^{111}–Lys^{112} and Lys^{112}–Asn^{113} bonds. When the enzyme was used in the continuous mode, however, it was found that the preference towards the Lys^{116}–Thr^{117} bond depended on the relative concentrations of both the CMP substrate and the likely enzyme-inhibiting intermediary products accumulated at steady state. Such concentrations were controlled by the enzyme-to-substrate concentrations as well as the substrate feed flow rate. The authors concluded that by controlling the operating parameters in the membrane reaction process, the enzyme hydrolysis pattern on various peptide bonds could be directed in a manner that could lead to the direct influence of the type of product composition obtainable.

This study underscored a fundamental aspect which has gathered little momentum: that the hydrolysis parameters have a way of "switching" the enzyme to hydrolyze a substrate in a particular manner. What was not confirmed was whether trypsin deviated from its narrow specificity in order to attack other peptide bonds formed by different amino acids from those for which it is known (namely, the carboxy terminals of Lys and Arg). Furthermore, although the mapping of a substrate like

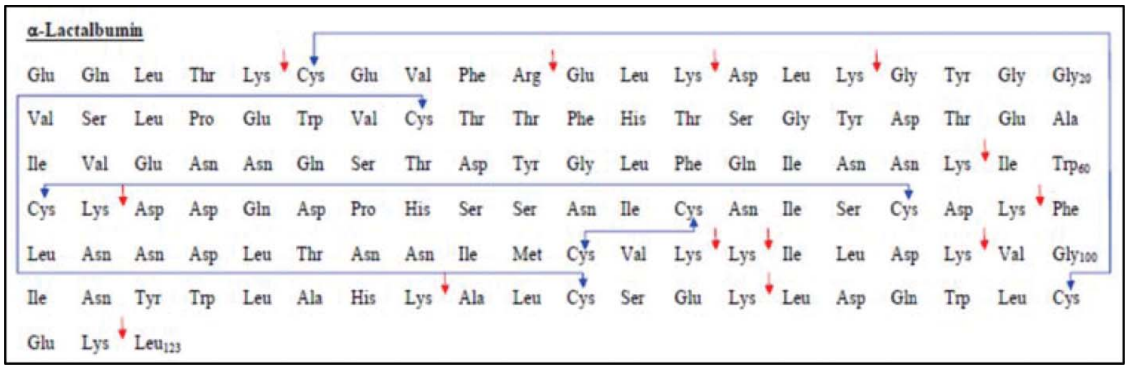

Figure 14. Amino acid sequence of bovine α-La showing the potential trypsin attack sites highlighted with a red down arrow. The intra-molecular disulphide bridges are also highlighted.

β-Lg clearly indicates the potential hydrolytic sites, it is evident that the protein tertiary structure as well as the presence of barrel and helix structures present potential restrictions to enzyme access and hence peptide bond attack. This information is important in order to improve the predictability and control of the hydrolysis process and products so as to help in the development of a process platform for possible "peptide design."

With the identified peptide bonds in β-Lg, it is also possible to predict the degree of hydrolysis, DH. In β-Lg, therefore, if the 18 peptide bonds were hydrolyzed by trypsin, it would be expected that $DH_{trypsin\text{-}max}$ would be 18 out of 161 which gives a value of 11.18%. However, since one peptide (Lys^{57}-Pro^{48}) bond is expected to resist trypsin hydrolysis, it is predicted that only 17 out of 161 peptide bonds would be scissile, resulting in a $DH_{trypsin\text{-}max}$ of 10.56%. In practice, this DH was found difficult to reach when whey proteins were hydrolyzed with trypsin, with values in the region of 9.5% being reported in the literature (Asselin et al., 1988; Amiot et al., 2004; Cheison et al., 2010) which translates to about 90% trypsin hydrolysis. Trypsin, therefore, produces larger peptides from whey proteins compared to other enzymes like Subtilisin, Protease N, Flavorzyme and Protease A which end up releasing higher amounts of free amino acids while hydrolysis WPI to DH values equal to or higher than 20% (Cheison et al., 2007a).

Hydrolysis has also been performed on HP-treated β-Lg using trypsin. Thus Chicon et al (2006a) studied the effect of hydrolysis time and pressure (0.1–400 MPa) on the proteolysis of β-Lg A with trypsin either under pressure or hydrolysis of β-Lg that was previously pressure treated. It was found out that pressurization, before or during enzyme treatments, enhanced tryptic hydrolysis of β-Lg. Trypsin degraded pressure-modified β-Lg and pressure-induced β-Lg aggregates, favoring proteolysis to the intermediate degradation products. Thus peptides identified were those for which trypsin was expected to release from β-Lg: Val^{15}-Arg^{40}, Val^{41}-Lys^{69}S_SLeu^{149}-Ile^{162}, and Val^{41}-Lys^{70}S_SLeu^{149}-Ile^{162}. As hydrolysis time progressed, the precursor peptides were further cleaved to yield peptides like: Val^{15}-Tyr^{20}, Ser^{21}-Arg^{40}, Val^{41}-Tyr^{60}, Trp^{61}-Lys^{69}S_SLeu^{149}-Ile^{162}, and Trp^{61}-Lys^{70}S_SLeu^{149}-Ile^{162}. Particularly, in the tryptic hydrolysates of pre-pressurized β-Lg, two other fragments linked by disulphide bonds: Lys^{101}-Arg^{124}S_SLeu^{149}-Ile^{162} and Tyr^{102}-Arg^{124}S_SLeu^{149}-Ile^{162}, were found. These corresponded to rearrangement products induced by SH/SS exchange between the free thiol group of Cys^{121} and Cys^{160}, that normally forms the disulphide bond Cys^{66}-Cys^{160}. HP-treatment suggests molecular re-arrangement and was proposed as an important tool in pre-hydrolysis protein treatment.

For the hydrolysis of β-Lg, in addition to substrate concentration and enzyme-to-substrate ratio, external parameters, like the hydrolysis pH and temperature, have to be considered (Christensen, 1949). Every enzyme has a specific pH optimum at which it works best. Lowering or increasing that value in the hydrolysis environment, leads to an influence on the enzyme activity. In the range between 7 and 9 trypsin seems not to be affected by a change of pH (Mota et al., 2006). Conversely, the pH has a strong influence on the solubility and viscosity of protein solutions. This is because the side groups of proteins are deprotonated at a high pH and protonated at a low pH to a high extend. In addition, β-Lg undergoes autolysis under high alkaline conditions (Christensen, 1949) which process would make enzymatic cleavage less controllable, and exaggerated.

Trypsin hydrolysis α-La

Whey protein hydrolysis seems to generally proceed with β-Lg being hydrolyzed more favorably than α-La. α-La is known to be susceptible to pepsin (Schmidt and van Markwijk, 1993), thermolysin (N'Negue et al., 2006; Otte et al., 2007b), Cardosin A (Barros et al., 2003) pepsin and papain (Schmidt and van Markwijk, 1993), chymosin (Miranda et al., 1989). Generally, it was found that α-La was more susceptible to pepsin than trypsin (Schmidt and Poll, 1991), a property which was exploited to develop an enzymatic method for its purification by selectively hydrolyzing β-Lg (Konrad and Kleinschmidt, 2008; Cheison et al., 2011d).

The potential trypsin attack sites in α-La are 13 out of the 122 peptide bonds. That translates to a total trypsin hydrolysis DH of 10.65%. α-La was slowly hydrolyzed by trypsin at its optimal conditions but rapidly by pepsin in either pure form or in whole whey. Their hydrolysates had molecular weights from about 8 kDa to less than 500 Da with the majority being 3–4 kDa (Pintado et al., 1999). The relative resistance of α-La to trypsin is possibly the reason behind the paucity of tryptic peptides reported in literature as compared to the litany of peptides from β-Lg. This is because earlier works with enzymes employed animal proteinases, including trypsin. Results suggested that in whey protein hydrolysis, trypsin attacks α-La immediately after completion of β-Lg digestion. Although the enzyme trypsin was reported to be poor in hydrolyzing α-La, hydrolysis at 37°C and pH 9 and at 50°C and pH 8 led to depletion of the protein in 15 min while hydrolysis at 37°C at a pH of 8 took 120 min to deplete (Mota et al., 2006). This showed that the resistance of the protein was milieu dependent. Our work has recently revealed that α-La resistance to trypsin was dependent on the milieu conditions under which hydrolysis was performed. Generally, hydrolysis at trypsin optimal conditions as well as at lower temperature and elevated pH led to selective digestion of β-Lg in whey proteins containing α-La. We found out that when hydrolysis was performed at elevated temperature and pH (50°C for example) the selectivity was lost leading to random hydrolysis of all the proteins (Cheison et al., 2011d). Aside from the reported susceptibility to pepsin, α-La, and not β-Lg, was selectively hydrolyzed by Protease A under acid pH conditions implying that the pH is a major factor in the protein susceptibility to enzyme digestion (Cheison et al., 2012).

Enzyme kinetics

Enzyme reaction is simplified as conversion of a substrate to a product. In protein hydrolysis, the substrate is invariably the peptide bond. In simplified enzyme hydrolysis models of proteins, the assumption is that the protein as a "whole" is the substrate. This is problematic because as seen already, enzymes like trypsin and chymotrypsin catalyze the breakdown of

specific peptide bonds in a protein. In its simplified form, the enzyme catalyzed reaction is represented by the reaction scheme shown in Equation (2.6).

$$E_{total} + S \underset{\leftarrow}{\overset{k_1}{\underset{k_{-1}}{\rightleftarrows}}} ES \xrightarrow{k_2} E + P \tag{2.6}$$

This over-simplification of the reaction scheme does not recognize the several transition steps through which the enzyme proceeds in converting the substrate to intermediates which are then converted, before the enzyme dissociates with it to start a new conversion cycle, to yield a product. In order to disambiguate the reaction kinetics, it is important to estimate the enzyme mass balance according to the scheme in Equation (2.7).

$$E_{total} + S \underset{\leftarrow}{\overset{k_1}{\underset{k_{-1}}{\rightleftarrows}}} ES \underset{\leftarrow}{\overset{k_2}{\underset{k_{-2}}{\rightleftarrows}}} EP \xrightarrow{k_3} E_{free} + E_{Inhibited} + E_{denatured} + P \tag{2.7}$$

The enzyme available at the initiation of the reaction (E_{total}) is partitioned into the free enzyme (E_{free}), the enzyme that is inhibited by the product and substrate ($E_{inhibited}$) and the denatured enzyme ($E_{denatured}$). The substrate conversion goes though the enzyme-substrate (ES) complex to the enzyme-product complex (EP) before dissociation to yield the product (P). However, enzyme hydrolysis of proteins should be appreciated as being more complex than a single-enzyme-single-substrate reaction. It is better appreciated as a multi-substrate reaction although this approach leads to a complex work-around of the classical Michäelis–Menten kinetics. Such an approach would also lead to a modification of Equation (2.6) according to Costa and Malcata (1994):

$$E_{total} + S_i \underset{\leftarrow}{\overset{k_{m,i}}{\underset{k_{-m,i}}{\rightleftarrows}}} ES_i \xrightarrow{k_{cat,i}} E + P_i, \quad i = 1, 2, 3, \ \ldots n \tag{2.8}$$

Where S_i and P_i are the corresponding substrate and product of the *i*th order, respectively, whereas *n* is the number of different peptide bond substrates present. In the case of trypsin hydrolysis of β-Lg, for example, this may be assumed to be 17. The quantity $k_{m,i}$ is the dissociation constant of the enzyme-substrate complex, ES_i, while $k_{cat,i}$ is the catalytic constant associated with the formation of the product, P_i (Costa and Malcata, 1994).

The rate of product formation is, therefore, best described by Equation (2.9):

$$r_i = \frac{\left(\frac{V_{max,i}}{K_{m,i}}\right) C_{S_i}}{1 + \sum_{j=1}^{n} \frac{C_{S_i}}{K_{m,j}}}, \quad i = 1, 2, \ \ldots n \tag{2.9}$$

Where r_i denotes the rate of production of product, P_i, and C_s denotes the molar concentration of substrate, S_i. Here $V_{max,i}$ is the reaction rate under saturation conditions of substrate (i.e., $V_{max,i} \equiv k_{cat,i}$ CE, where CE is the total concentration of enzyme active sites) and $K_{m,i}$ is the classical Michaelis–Menten constant.

In order to appreciate the likely role played by the product in the inhibition of the enzyme, the Michäelis–Menten relationship becomes that represented in Equation (2.10).

$$V = \frac{k_2[E_0][S_0]}{K_m\left(1 + \frac{[P_i]}{K_{[pi]}}\right) + [S_0]} \tag{2.10}$$

The concentration of the product inhibitor (P_i) and the corresponding Michäelis–Menten constant are added to the classical equation and indicate competitive product inhibition. Under steady-state kinetics, applying the law of mass action on the enzyme mass balance gives the relationship in Equation (2.11) (Connors, 1989).

$$\frac{d[ES]}{dt} = k_1[E][S] - (k_{-1} + k_2)[ES] = 0 \tag{2.11}$$

Where the dissociation constant is defined as in Equation (2.12):

$$K_m = \frac{k_{-1} + k_2}{k_1} \tag{2.12}$$

The rate of substrate disappearance may be used instead of the rate of product formation which is given by Equation (2.13), especially for proteins since disparate peptides are formed all at the same time.

$$\frac{dP}{dT} = \frac{k_2[E_0][S]}{K_m + [S]} \tag{2.13}$$

The reaction rate is determined by the irreversible stage (Equation 2.14), which considers the DH in the rate of reaction (Márquez and Vázquez, 1999):

$$r = s_0 \frac{d(DH)}{dt} = k_2[ES] \tag{2.14}$$

Equation (2.14) introduces the DH in the rate of substrate disappearance at steady state, a prominent index used in monitoring protein hydrolysis. Regarding the enzyme, the kinetic equation for the reaction would be given by Equation (2.15).

$$-\frac{d[E_{total}]}{dt} = k_3[E][ES] \tag{2.15}$$

By dividing Equation (2.14) by Equation (2.15), the ratio in Equation (2.16) is obtained:

$$-s_0 \frac{d(DH)}{d[E_{total}]} = \frac{k_2}{k_3[E]} \tag{2.16}$$

Márquez & Vázquez (1999) have shown that the kinetic model for the DH can be fitted using Equation (2.17):

$$\frac{d(DH)}{dt} = a\exp[-b(DH)] \tag{2.17}$$

Where:

$$a = \frac{k_2[E_{initial}]}{s_0} \quad (2.18)$$

While

$$b = \frac{k_3 K_m}{k_2} \quad (2.19)$$

The rate of reaction, therefore, is given by Equation (2.20):

$$r = k_2[E_{total}]\exp\left[-\frac{k_3 K_m}{k_2}(DH)\right] \quad (2.20)$$

The total DH of protein hydrolysates can be approximated using a number of methods such as the pH-stat and osmometry (Adler-Nissen, 1986; Cheison et al., 2009). In addition, methods that rely on the amino group (NH_2) reaction chemistry are used. These include the 2,4,6-trinitrobenzene 1-sulphonic acid (TNBS) (Adler-Nissen, 1979), ninhydrin (Schwartz and Engel, 1950; Doi et al., 1981), and the *O*-phthalaldehyde (OPA) (Church et al., 1985). The pH-stat method is linearly correlated with the more laborious chromogenic methods (Spellman et al., 2003; Cheison et al., 2009), hence can be adopted for DH calculation for hydrolysis performed at alkaline and acid pH conditions (Cheison et al., 2012). Meanwhile, enzyme kinetics correlate well with the DH (Camacho et al., 1998).

The need to monitor the products of hydrolysis in order to help develop a multi-substrate reaction model for proteins is an urgent one. Since many reaction products are released by the enzyme almost at the same time, it is important to attempt the studies under conditions in which the hydrolysis pattern elucidation, rather than the "speed" of reaction is considered (Frenzen and Maini, 1988; Gonzàlez-Tello et al., 1994; Shi et al., 2005). Meanwhile, urgent analytical methods are invited to help isolate, identify and/or analyze the products. Until that time that the products can be analyzed using competent methods, the DH shall remain the method of choice for the estimation of enzyme kinetics with the possibility of including a mass spectrometric method to identify the products (Tauzin et al., 2003; Greis, 2007).

Factors that influence enzyme hydrolysis of proteins

Protein hydrolysis is performed in an environment whose temperature and pH is regulated. Most of the conditions of choice encountered in the literature review are those suggested by the manufacturer of the enzyme. These are generally conditions under which the enzyme hydrolysis is optimal, generally maximum.

Temperature

Focus has hitherto been on the influence of the hydrolysis temperature on the activity and stability of the enzyme (Fig. 15). However, the impact of temperature supersedes its activation and/or denaturation of the enzyme and includes influence on substrate molecular structure and susceptibility of enzyme attack. For example, the influence of the pH and temperature on the structure of β-Lg is well characterized (Pessen et al., 1985). Although it exists in dimeric forms under the physiological conditions of milk, it aggregates into octamers (especially for genetic variant A) at pH 4.65, and the dimeric form dissociates to monomers upon warming above 40°C or at pH values above 7.5, the so-called Tanford transition (Tanford et al., 1959). The conformational change brought about by the Tanford transition provides a structural basis for a variety of pH-dependent chemical, physical and spectroscopic phenomena (Qin et al., 1998); prominently, there is a 5 to 10% increase in protein hydration and a loosening of the interior packing of β-Lg (Taulier and Chalikian, 2001), a property which we found to account for increased hydrolysis by trypsin (Cheison et al., 2011d) and chymotrypsin (Lisak et al., 2013).

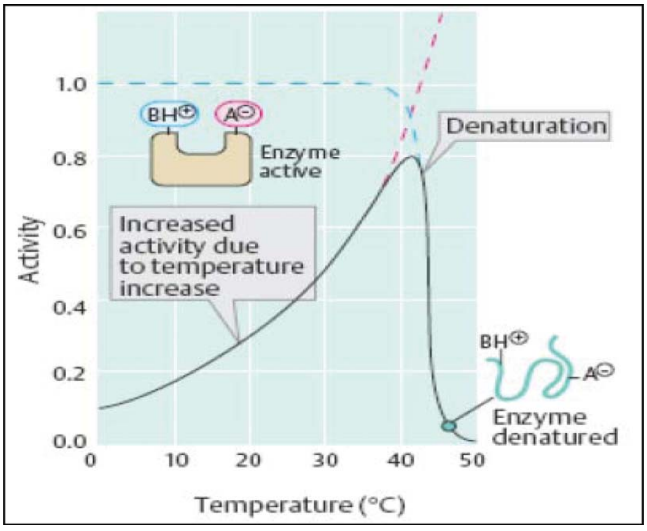

Figure 15. Influence of temperature on enzyme activity showing regions of low activity below and above the temperature optimum.

Meanwhile, the influence of enzyme type, pH and temperature during hydrolysis on enzyme kinetics during hydrolysis of whey protein was investigated by Camacho et al (1998). The temperature and pH influence on β-Lg lead to conformational changes (Fig. 3). The Tanford transition, which occurs at pH 7.51 and/or 40°C (Tanford et al., 1959), leads to increased hydration and loose protein interior structure (Taulier and Chalikian, 2001). Therefore, the accessibility of cleavage sites to the enzyme is improved. In contrast, molecular aggregation occurs at high temperature and low pH. Most of the molecules are buried, which reduces their accessibility to hydrolysis. Furthermore, the β-structure of β-Lg is stable at acidic pH but less stable at alkaline pH, a factor which likely increased its resistance to hydrolysis under acid pH (Cheison et al., 2012). In contrast, the α-helix is more stable at alkaline pH (Johnson, 1988).

Many reports demonstrate the resistance of β-Lg to proteases like pepsin (Chen et al., 1993; Schmidt and van Markwijk, 1993; Guo et al., 1995; Iametti et al., 2002) and Protease A (Cheison et al., 2012). Using limited proteolysis (by lowering the temperature) with immobilized trypsin, Chen et al. (1993) found two large polypeptide fragments, f(48–101) and f(41–100), which were thought to be from the core domain of β-Lg and resistant to trypsinolysis. Compared to native β-Lg, these fragments had similar β-strands but no α-helices. Molinari

et al. (1996) found the same phenomenon while Iametti et al. (2002) sub-denatured β-Lg during thermal treatment and hydrolyzed it with trypsin. They obtained fragments in the region of f(41–70). Comparing these reports, it seems that at high temperature, α-La (N'Negue et al., 2006) and β-Lg are relatively more hydrolysable, a factor we confirmed with trypsin hydrolysis at 50°C (Cheison et al., 2011d).

The temperature also influences the protein solubility. On the one hand, higher temperature leads to an increase of the collision energy of the molecules. Conversely, it causes reactions of the side groups, which can form cross-linkages within and between the peptide chains. These cross-linkages may reduce the protein solubility (Walstra et al., 2006). Generally, the protein solubility increases with temperatures between 40°C and 50°C, but if the temperature is too high, the protein will denature (Pelegrine and Gasparetto, 2005), which leads to aggregation.

With regard to the enzyme hydrolysis and calculation of the DH based on the pH-stat method (Equation (2.2) for hydrolysis under alkaline pH and Equation (2.3) for hydrolysis under acid pH) the only parameter that has to be derived is α (the dissociation constant of either the α-amino or carboxyl groups). The value of α is dependent on the pK value. However, the value of pK is not a constant; it varies with the nature of the terminal amino acid, the chain length of the peptide and the temperature (Steinhardt and Beychok, 1964), but is relatively independent of the substrate. The first two points can be neglected, because it is always the same substrate and the same enzyme and, therefore, there should be nearly the same size of fragments, and the terminal amino acids also should not vary so much. But the variation of the pK with temperature is quite large, this is based on the ionization enthalpy of the amino group, that is in the average around 45 kJ/mole (Steinhardt and Beychok, 1964).

Using the Van't Hoff equation (Equation (2.21))

$$\Delta G^{\circ} = -RT \times \ln(K_s) \tag{2.21}$$

and the Gibbs–Helmholtz equation (Equation (2.22)

$$\Delta G^{\circ} = \Delta H^{\circ} - T \times \Delta S \tag{2.22}$$

and with the additional knowledge, that

$$pK_s = -\lg(K_s) \tag{23}$$

the following combined equation is obtained:

$$pK = \frac{\Delta H^0}{2.303 \times RT} - \frac{\Delta S^0}{2.303 \times R} \tag{2.24}$$

Where:

$\Delta G^0 =$ free energy
$\Delta H^0 =$ enthalpy (45 kJ/mol)
$\Delta S^0 =$ entropy
$R =$ gas constant (8.314472 J/mole/K)
$T =$ temperature (K)
$K_s =$ acid dissociation constant

With the help of Equation (2.11), the pK can be calculated. Generally, a temperature change of 10°C or 10 K in hydrolysis leads to a pK change of about 0.23 pH units (Adler-Nissen, 1982) and, therefore, to a change of the factor α. A huge advantage of the described pH-stat technique is, that one can continuously follow the DH during the reaction.

The pH

The effect of pH on enzymes varies but is prominently represented by the fact that each enzyme has an optimum pH-range and is denatured on either side of that pH (Fig. 16).

Generally, the proteins are more soluble in acid or alkaline pH range, because the excess of the same charges (+/−) repulse among the molecules and this leads to a high solubility. On the other hand, if the pH is so high or so low then it denatures the secondary and tertiary structure, the protein unfolds and more hydrophobic groups are exposed, which leads to a reduction of water binding. In agreement with multiple researchers (Kakalis & Regenstein, 1986; de Wit, 1989; Wong et al., 1996) the pH with the lowest solubility is the isoelectric point, pI (Fig. 17b&d). That is, because the electrostatic forces are at a minimum and, therefore, the protein–protein interactions increase, aggregates build and the interaction of water and protein molecules decrease (Pelegrine & Gasparetto, 2005). The pI for β-Lg lies around pH 5.2. This pI region should be avoided during the experiments unless the protein is to be protected using the pI aggregation since the solubility of the substrate has an influence on the enzyme access and hence hydrolysis. The better a protein is dissolved the easier it is for the enzyme to access the preferential cleavage sites and to hydrolyze them.

Buffer salt type and concentration

The interactions between proteins and the solvent media are complex (see Fig. 17a&c). A quantitative understanding of the forces in aqueous solution that control chemical processes and give rise to biological structure is essential. Specifically, an ion may affect the enzyme by playing the role of a substrate, a cofactor, or an inhibitor. The theory of acid-base in enzyme catalysis requires the exchange of protons under a favorable medium. Relative to the strength of water–water interactions in

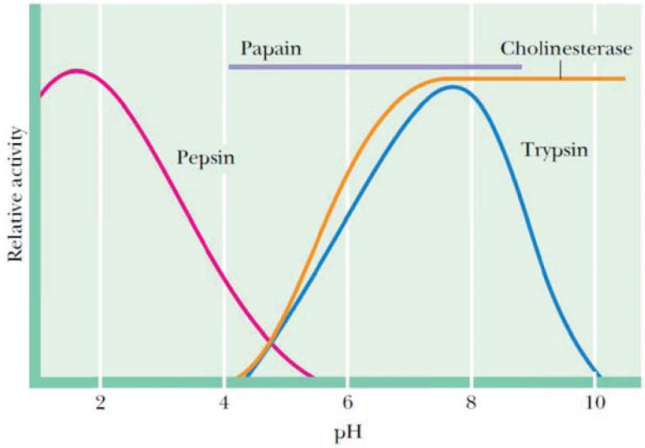

Figure 16. Enzyme sensitivity to pH showing the optimum pH for trypsin as compared to other common enzymes (source Garrett and Grisham (2007)).

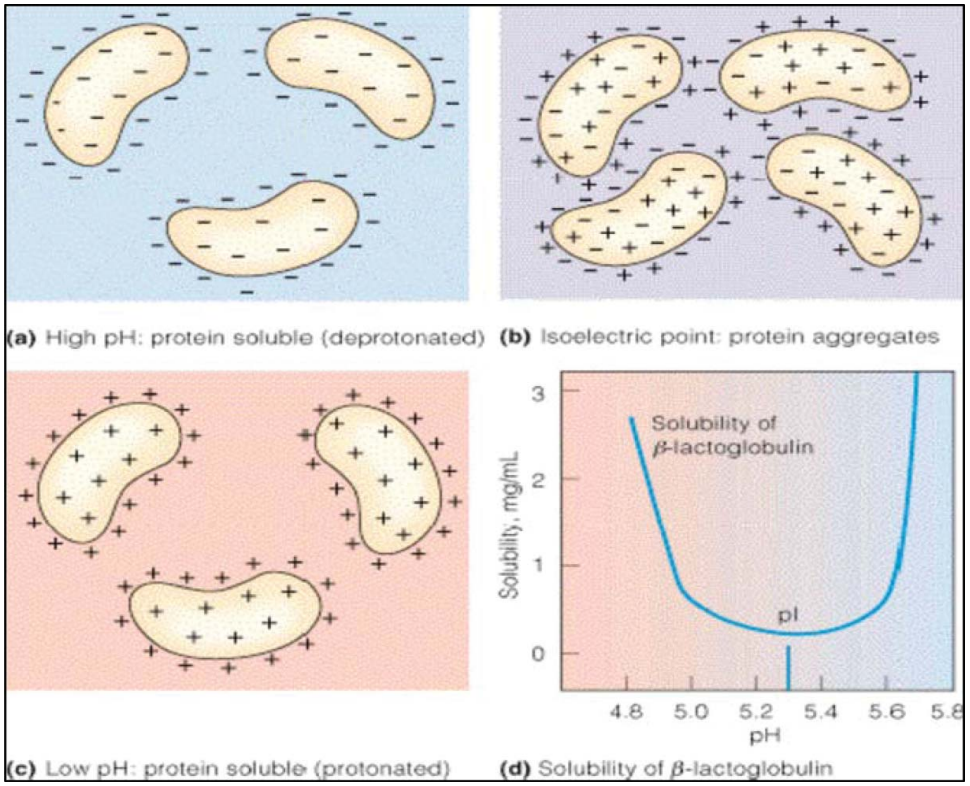

Figure 17. Influence of the environmental charge properties on protein solubility at high and low pH (Adopted from Mathews & van Holde (1995)).

bulk solution, small ions of high charge density (kosmotropes) bind water molecules strongly, whereas large monovalent ions of low charge density (chaotropes) bind water molecules weakly. Buffer cations show a less dominant effect relative to anions of the same charge density, because anions are more polarisable and hydrate more strongly. An ion can not only compete with the enzyme for the water surrounding the enzyme molecule but also penetrates into the water layer to get direct contact with the enzyme and to interact with it (Broering and Bommarius, 2005).

A protein dissolved in aqueous solution has on its surface many hydrophilic and polar moieties, especially charged groups, responsible for hydration and interactions with the ions in solution. These groups can be mainly divided into two entities: the chaotropic amide and amino groups and the kosmotropic carboxylic groups. Chaotropic anions are water-structure breakers and destabilize proteins. Kosmotropic anions are polar waterstructure makers and generally stabilize proteins. The effect of ions on enzyme activity during protein hydrolysis is not so straightforward. Ions may have strong interactions with the functional groups on the surface of the enzyme, especially those in the enzyme active site. This will trigger a change in the enzyme active site both chemically and physically, resulting in a modification in the enzyme catalytic activity and even its catalytic mechanism.

Kalisz et al. (1997) have shown that increasing the acetate or phosphate buffer concentration from 50 mM to 1 M caused a three- and ten-fold increase in the thermal stability of *Penicillium amagasakiense* glucose oxidase at pH 6.0 and 8.0, respectively, and a 100-fold stabilization at pH 7.0. A recombinant *Aspergillus fumigatus* phytase demonstrated better thermostability at 65 and 90°C in acetate than in citrate buffer at pH 5.5. The enzyme had a greater heat tolerance in 10 mM than in 200 mM at 65°C. The heat stability of the enzyme originates from its ability to refold completely into the native-like, fully stabilizing in the native protein, which coulombic interactions are reduced at low ionic strength (Dominy et al., 2002). These two examples underscore the role of buffer concentration and salt types on the protein stability. Protein solubilities are affected to dissimilar extents by the ionic content of the buffer, some being soluble at higher salt concentration than others (Fig. 18).

Although the molecular dimer-monomer dissociation of β-Lg was demonstrated to be dependent on, and to be favored by, the pH (>pH 7.5) and temperature (>40°C) (Tanford et al., 1959; Pessen et al., 1985), the effect of the buffer concentrations on the equilibrium between dimers and monomers is still unclear. Decreased ionic strength and temperature, for example favors oligomerization of β-Lg at pH 4.65 (Verheul et al., 1999). Dimerization is favored by changes in the pH towards the pI as well as an increase in the ionic strength (Vardhanabhuti and Allen Foegeding, 2008), and is influenced by protein concentration.

In addition, Hydrolysis under buffer produced trypsin-specific peptides, numerous chymotrypsin-like nonspecific peptides but no disulphide-linked peptides. Trypsinolysis shifted to the N-terminal region of lysine under some conditions (Cheison et al., 2011c).

Tris-H^+ possesses a large deprotonation enthalpy. Upon eventual protonation or deprotonation of the protein that might occur during conformational changes and Ca^{2+}-binding, the measured heat exchange (ΔH_{exp} values) also includes a contribution for the transfer of those protons from, or to, the components of the Tris-HCl buffer. Therefore, the impact of the

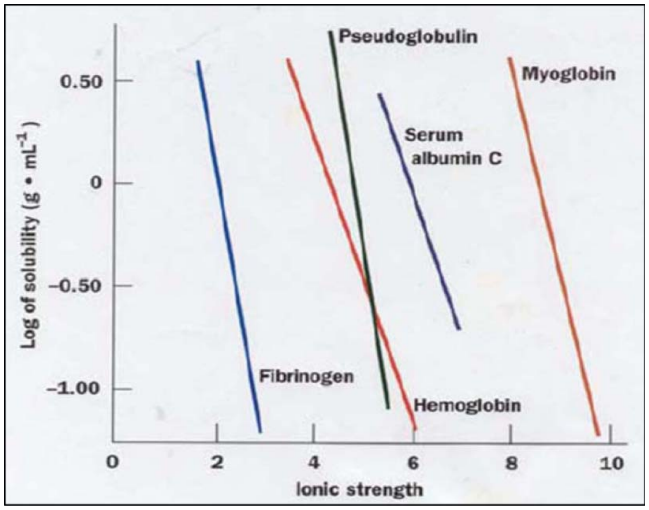

Figure 18. Influence of salt concentration on protein solubility showing that fibrinogen and myoglobin are more and less sensitive to salt concentration, respectively.

deprotonation or protonation of the buffer components is measured to eventually correct the observed heat exchange (ΔH_{exp}). Of the resulting thermodynamic parameters (ΔH, ΔS, ΔG, ΔC_p), derived for the conformational transition and for the Ca^{2+}-binding of the α-La, especially the heat capacity increments (ΔC_p values) are closely related to changes in the exposure of hydrophobic surface.

A mixture of α-La and saponins when hydrolyzed using trypsin showed that Saponin increased the tryptic-hydrolysis level of α-La only at relatively high phosphate buffer concentrations (greater than or equal to 0.05 M)

Duration of hydrolysis

The time an enzyme takes to digest a protein is directly linked to the sizes of the peptides, and consequently the diversity of the products, released. Time has been studied as a variable of protein hydrolysis and shown to influence the peptide composition of whey protein hydrolysates produced using trypsin and protease 2A (Pintado et al., 1999). The rate of protein depletion, rate of peptide formation and rate of enzyme denaturation (Costa and Malcata, 1994; Margot et al., 1997) were all shown to be time-dependent. It is generally agreed that the first few minutes of hydrolysis constitute a region of critical enzyme kinetics which may be used to monitor not only the initial attack sites and enzyme hydrolysis progress but the kinetics of those first hydrolysis points (Cheison et al., 2010; Cheison et al., 2011a; Cheison et al., 2011b). It remains unclear, however, whether the enzyme attacks the same peptide bonds under different milieu conditions or there is a shift not only on the kinetics but also attack sequence and peptide bond preference. As a means to steer the enzyme, it is important to unravel these first attack sites in order to determine when and whether a process can be stopped in order to tailor the products and/or avoid product inhibition of the enzyme.

Presence of the product

Enzymes release products whose interaction with it might be considered as inhibitory if the separation between the enzyme (E) and product (P) in the EP-complex is either delayed or fastidious. It is generally recognized that carbohydrate hydrolysis is beset by product inhibition as is the case with lactose (Demirhan et al., 2007; Hu et al., 2010), cellulose (Bezerra et al., 2011) and lignocellulose (Andrić et al., 2010). Recently, the impact of product inhibition during whey protein (Cheison et al., 2007a) and sesame cake protein hydrolysis (Demirhan et al., 2011) was reported. It was also proposed that there existed an irreversible serine-protease "inhibitor" in whey proteins (Gonzàlez-Tello et al., 1994) which was probably a case of severe product inhibition. Product inhibition was also reported for gluten hydrolysis (Apar and Özbek, 2010). Understandably this nuisance was a motivation behind the emergence of enzyme immobilization and use of membrane reactors (Cheftel et al., 1971; Hatzinikolaou et al., 2005; Cheison et al., 2006b; Trusek-Holownia, 2008). Nevertheless, it remains underestimated as a deterrent in enzyme hydrolysis progress and must be revisited as a potential influence to enzyme kinetics.

Bioactive and functional peptides

Hydrolysis of proteins not only yields improved hydrolysates in their functional properties. Bioactivity is also augmented due to the release of peptide fragments from a parent, bioactively inactive, protein. Although many enzymes were used to produce bioactive peptides from whey proteins, tryptic hydrolysates are of interest because of the enzyme specificity which enables the production of predictable peptides under controlled conditions. Many different enzymes were used and the hydrolysates displayed various bioactive properties. For example, Choi et al. (2009) showed that WPC hydrolysates were produced using the proteases such as neutrase (WPH-N), alcalase (WPH-A), flavorzyme (WPH-F) and protamex (WPH-P).

No significant inhibitory activity was detected toward the tumor cell lines tested in this study with WPC, WPH-N, WPH-F, and WPH-P, whereas WPH-A showed cytotoxic effects toward the MCF-7, A-549 and SK-MES-1 cell lines. WPH-A showed cytotoxicity toward these tumor cell lines, in a hydrolysis time-dependent manner for up to six hours, after which the cytotoxicity was reduced. The inhibitory effects of WPH-A on the normal human lung cell line MRC-5 was also determined and revealed that WPH-A was more cytotoxic toward tumor cell lines (A-549 and MCF-7) than normal cells (MRC-5).

Bioactive peptides from β-Lg

In its native form, β-Lg has no known bioactivity beyond the postulated role as a transporter of small hydrophobic molecules like retinol, vitamin D and cholesterol and some fatty acids and palmitate (Pérez and Calvo, 1995; Madureira et al., 2007). This was deduced because of its structural similarity to other proteins of the lipocalin family which are known transporters of small molecules. Following hydrolysis with enzymes, however, β-Lg, like many other proteins with little or no known innate bioactivity, undergoes a dramatic tropho-functional activation due to the release of peptides from its protein matrix (Meisel and Bockelmann, 1999). Those peptides were characterized to possess potential bioactive properties based on their structures as well as proven in various tests (Table 2.1). Hence β-Lg released peptides, with angiotensin-I-converting enzyme (ACE) inhibitory properties following hydrolysis using trypsin (Mullally et al., 1997b), Protease N (Ortiz-Chao et al., 2009) and thermolysin

Table 2.1. Bioactive peptides obtained from β-Lg hydrolysis using various enzymes.

Fragment	Sequence	Enzyme used	Bioactivity	Reference
f(42–46)	Tyr-Val-Glu-Glu-Leu	Thermolysin	Antioxidant	(Hernández-Ledesma et al., 2006)
f(145–149)	Met-His-Ile-Arg-Leu			
f(19–29)	Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile			
f(15–20)	Ala-Gly-Thr-Trp-Tyr	Pepsin	Antimicrobial	(Pellegrini et al., 2001)
f(25–40)	Ala-Ala-Ser-Asp-Ile-Ser-Leu-Leu-Asp-Ala-Gln-Ser-Ala-Pro-Leu-Arg			
f(78–83)	Ile-Pro-Ala-Val-Phe-Lys			
f(92–100)	Val-Leu-Val-Leu-Asp-Thr-Asp-Tyr-Lys			
f(102–105)	Tyr-Leu-Leu-Phe	trypsin, chymotrypsin, pepsin	Opioid	(Antila et al., 1991)
f(146–149)	His-Ile-Arg-Leu	chymotrypsin	Opioid, anti-hypertensive	(Yamauchi et al., 2003, Yoshikawa et al., 1986)
f(71–75)	Ile-Ile-Ala-Glu-Lys	trypsin	Hypocholesterolemic	(Nagaoka et al., 2001)
f(9–14)	Gly-Leu-Asp-Ile-Gln-Lys			
f(142–146)	Ala-Leu-Pro-Met-His			
f(41–60)	Val-Tyr-Val-Glu-Glu-Leu-Lys-Pro-Thr-Pro-Glu-Gly-Asp-Leu-Glu-Ile-Leu-Leu-Gln-Lys			

(Hernández-Ledesma et al., 2006). The peptides responsible for the bioactive property were different, owing in part to the enzyme employed for its production. Thus, the peptides from the sequence corresponding to f(142–148), f(36–42), and f(58–61) were identified in those studies, respectively. Those peptides have potential to maintain blood pressure integrity.

In addition, β-Lg peptides demonstrated immunomodulating effects using murine splenocytes cultured in the absence and presence of concanavalin A (ConA) (Saint-Sauveur et al., 2008). Evidence of immune-modulation was offered by the fact that the peptide fractions all stimulated the proliferation of splenocytes in the presence and absence of ConA, with the exception that WPI inhibited proliferation at high concentrations in the presence of ConA. The peptide fractions significantly stimulated the secretion of IL-2 and IFN-γ. Peptides also possessed the potential to lower cholesterol levels (Nagaoka et al., 2001) as well as possessing antioxidant (Hernández-Ledesma et al., 2005) and mineral-binding (Baumy and Brule, 1988) properties. Of interest also is the property which was noted in peptides with opioid agonist properties. Thus β-lactotensin (β-LT: His-Ile-Arg-Leu) is an ileum-contracting peptide derived from residues f(146–149) of bovine β-Lg (Yamauchi et al., 2003). It was reported that β-Lg possessed some anti-microbial domains within its protein matrix (Pellegrini et al., 2001) as a result of trypsin digestion. Thus f(15–20, Val-Ala-Gly-Thr-Trp-Tyr), f(25–40, Ala-Ala-Ser-Asp-Ile-Ser-Leu-Leu-Asp-Ala-Gln-Ser-Ala-Pro-Leu-Arg), f(78–83, Ile-Pro-Ala-Val-Phe-Lys) and f(92–100, Val-Leu-Val-Leu-Asp-Thr-Asp-Tyr-Lys) were found to exert bactericidal effects against the Gram-positive bacteria only. Bioactivity against *Escherichia coli* JM103 was also reported in another study (Pihlanto-Leppälä et al., 1999). It is clear from the literature review that the composition of peptides, their efficacy in bioactivity assay and the structure–function relation are closely tied to the protein source, its molecular structure (and amenability to structural changes induced by temperature treatment) and the type of enzyme used to produce the peptides. In addition, the DH and/or time of hydrolysis determine the release of the bioactive peptides because of the kinetics of release of the peptides.

Bioactive peptides from α-La

Peptides and hydrolysates of α-La with antimicrobial properties have been reported (Table 2.2). Bactericidal peptides against Gram-positive bacteria were obtained after α-La digestion with trypsin and chymotrypsin, but not with pepsin (Pellegrini et al., 1999). The α-La tryptic peptides were identified as pentapeptide sequence f(1–5) and two peptides linked by a disulphide bridge corresponding to the amino acids f(17–31)-S_S-f(109–114). Digestion with chymotrypsin yielded one bactericidal peptide composed of two polypeptides linked by a disulphide bridge corresponding to the amino acids f(61–68)-S_S-f(75–80) of α-La. Disulphide linkage between the α [corresponding to peptide f(61–68)] and β [corresponding to peptide f(75–80)] component was necessary for bactericidal activity as no antimicrobial activity was observed with mixtures of individual peptides. The overall structure of the peptides may, therefore, be of critical importance for their action on bacterial membranes. The peptides lacked fungicidal activity against *Candida albicans*, while the bacterium *Bacillus subtilis* was most susceptible.

In a separate study both pepsin and trypsin released peptides from α-La which inhibited the growth of *E. coli* JM103 upon incubation with peptides-enriched basal medium, at 37°C. The peptide concentration was 25 mg/mL, whereas unhydrolyzed α-La did not inhibit growth at a concentration of 100 mg/mL (Pihlanto-Leppälä et al., 1999). Lack of antimicrobial activity in peptic hydrolysates during the former study may have been caused by low assay dosage ($5.5 \times 10^{-8} - 1 \times 10^{-6}$mol) implying a dose-dependent effect, low sensitivity of method employed, or microbe specificity. In contrast, α-La hydrolysates obtained with alcalase (EC 3.4.21.62), did not have antimicrobial activity and promoted the bioluminescent activity of *E. coli* JM103 compared with the same quantity of unhydrolyzed protein (0.1 g/mL). The DH of these hydrolysates was 30.4% compared to pepsin 12.6% and trypsin 11.6%.

Both pepsin and trypsin hydrolysates showed inhibition of bioluminescent activity of 31.8% and 38.0%, respectively, at a concentration of 0.05 g/mL. This implies that pepsin and trypsin have higher specificity in releasing peptides from α-La possessing antimicrobial activity. Accordingly, the DH contributed to the antimicrobial activity of the protein, but a higher DH did

Table 2.2. Bioactive peptides obtained from α-La hydrolysis using various enzymes.

Bioactivity	Peptide sequence	Enzyme used	Reference
Immune-modulating	Gly-Leu-Phe	Trypsin	Jaziri et al. (1992)
Antimicrobial	Glu-Gln-Leu-Thr-Lys	Trypsin/Chymotrypsin	Pellegrini et al. (1999)
	Gly-Tyr-Gly-Gly-Val-Ser-Leu-Pro-Glu-Trp-Val-Cys-Thr-Thr-Phe	Trypsin	
	Ala-Leu-Cys-Ser-Glu-Lys	Chymotrypsin	
	Cys-Lys-Asn-Asp-Gln-Asp-Pro-His		
	Ile-Ser-Cys-Asp-Lys-Phe		
Antiviral	[a]3-HP-α-La (modified)	Trypsin, Chymotrypsin, Pepsin	Oevermann et al. (2003)
Opioid	Tyr-Gly-Leu-Phe	Pepsin	Antila et al. (1991)
Mineral binding	Not identified	Trypsin, Alcalase, Flavorzyme, Papin	Kim et al. (2007b)
Antiulcerative/growth stimulating	Ile-Trp-Cys-Lys-Asn-Asp-Gln-Asp-Pro-His-Ser-Ser-Asn-Ile-Cys-Asn-Ile-Ser-Cys-Asp-Lys-Phe-Leu-Asn-Asn-Asp-Leu-Thr-Asn-Asn-Ile-Met-Cys-Val-Lys	Endopeptidase lysine C	Kanda et al. (2007)
Anti-hypertensive	Tyr-Gly-Leu ($IC_{50} = 409$)	Trypsin	Pihlanto-Leppälä et al. (2000)
	Val-Gly-Ile-Asn-Tyr-Trp-Leu-Ala-His-Lys ($IC_{50} = 327$)	Pepsin	Mullally et al. (1997a)
	Trp-Leu-Ala-His-Lys ($IC_{50} = 77$) Tyr-Gly-Leu-Phe ($IC_{50} = 733$)		
	Leu-Lys-Gly-Tyr-Gly-Gly-Val-Ser-Leu-Pro-Glu-Trp ($IC_{50} = 83$)	Pepsin	Mullally et al. (1997a); Otte et al. (2007a)
	Tyr-Gly-Gly-Val-Ser-Leu-Pro-Glu-Trp ($IC_{50} = 16$)	Trypsin	
	Gly-Val-Ser-Leu-Pro-Glu-Trp ($IC_{50} = 30$)	Chymotrypsin	
	Val-Ser-Leu-Pro-Glu-Trp ($IC_{50} = 57$)	Elastase, PTN 3.0S, Corolase, Thermolysin, Proteinase K, [b]BLP	

[a]3-HP: 3-hydroxyphthalic anhydride, [b]BLP: β protease.

not necessarily correspond to a higher antimicrobial activity. Hydrophobic properties, together with an excess of positive charges near neutral pH which have been postulated to be important for bactericidal activity, were found to be not crucial for bactericidal activity of peptides under the test conditions (Pellegrini et al., 1999).

The combination of peptide fractions containing α-La and glycomacropeptide (GMP) with the same concentration of 0.25 to 0.05 mg/mL showed a synergistic effect in inhibiting the association of enteropathogenic *Escherichia coli* EPEC, *Salmonella Typhimurium*, and *Shigella flexneri* with intestinal cells and may thus prevent infection (Brück et al., 2006). GMP inhibited association of the EPEC and *Salmonella* with CaCo-2 cells by an effective mechanism based on its sialic acid substructure which would survive the digestion, but the mechanism by which α-La exerted its activity remains unclear. It is possible that the whey product disturbs the cellular metabolism by increasing cell permeability, owing to the peptide having a charge property opposite to that of the cell membrane components. This would cause the bacteria to consume more adenosine-5′-triphosphate (ATP) in order to maintain normal membrane integrity and functions. ATP affects the protein synthesis mechanism of the bacterial cell, resulting in a lower amount of luciferase enzyme (EC 1.13.12.7) and, thus, lower bioluminescence emission in bacteria incubated with the whey peptides (Pihlanto-Leppälä et al., 1999).

Recently, the *in vitro* digestion of goat whey proteins using human gastric and duodenal juice resulted in antibacterial peptide profiles different from that obtained using commercial enzymes (Almaas et al., 2008). The protein α-La was partially degraded by human enzymes, while treatment with commercial enzymes fully degraded the protein. Its hydrolysates obtained with both human gastric juice and human duodenal juice strongly inhibited *Listeria monocytogenes* at 0.3 to 0.6% of protein concentration in the bacterial culture media. Undigested goat whey and hydrolysates digested with human gastric juice demonstrated no significant effect in inhibiting *L. monocytogenes*. This indicates that antibacterial caprine whey hydrolysates are mainly obtained in the duodenum during digestion. In the duodenum, tryptic digestion occurs under alkaline conditions, while in the stomach digestion is by pepsin under acidic conditions. Listeriosis, a bacterial infection occurring primarily in newborn infants, elderly patients, and patients who are immune-compromised may be controlled by these peptides. More work is required to identify the involved peptides and their mode of action.

The overall feature of helicity, charge distribution and amphiphilic properties have been associated with antimicrobial peptides (Pihlanto-Leppälä et al., 1999). It is generally assumed that these amphiphilic peptides disturb the cytoplasmic membrane of micro-organisms.

Many antimicrobial peptides have hydrophobic properties together with an excess of positive charges near neutral pH of

the bactericidal assay (Kagan et al., 1994; Pellegrini et al., 1992), converse to antimicrobial peptides derived from α-La that are negatively charged. This may explain why they were only weakly active against Gram-negative bacteria whose outer membranes contain a negatively charged lipopolysaccharide as a major component. The overall structure of the peptides may also be of critical importance in their action on bacterial cell membranes. Disulphide bonds between cysteines in different polypeptide chains are crucial in ordering structure of peptides possessing bactericidal activities (Pellegrini et al., 1999).

It can be postulated that generation of bactericidal peptide fractions by physiological endopeptidases of the mammalian gastro-intestinal tract after ingestion of α-La containing diet could occur, given that such peptides have been produced with human enzymes. These fractions are without doubt more important as antimicrobial agents compared to the intact α-La. Identification of these peptides and concentrations of peptides required to impart antimicrobial activity under physiological colon conditions should be investigated before their efficacy is fully claimed.

Several peptide fragments with antiherpetic activity were obtained by proteolytic digestion of α-La using trypsin, chymotrypsin, and pepsin (Oevermann et al., 2003). Digestion of α-La was done for x hours at E/S 10%, temperature 37°C, and pH 7.8 for trypsin and chymotrypsin and pH 2.0 for pepsin. Peptides with ACE inhibitory activity have been obtained through hydrolysis of α-La (Table 2.2) with various enzymes, namely, trypsin, chymotrypsin, pepsin, elastase (EC 3.4.21.36), PTN 3.0S, Corolase (E.C. 3.4.21.4), thermolysin (EC 3.4.24.27), proteinase K (EC 3.4.21.14), and BLP (EC 3.4.21.19) (Mullally et al., 1997a; Otte et al., 2007a; Otte et al., 2007b). Hydrolysis of α-La by pepsin and trypsin or their combination showed high ACE inhibition (IC_{50}) values of more than 84% (Mullally et al., 1997a). Thermolysin is particularly good in releasing potent ACE inhibitory peptides on hydrolyzing α-La (Otte et al., 2007b) as the enzyme preferentially cleaves the aromatic or hydrophobic Ile, Leu, Phe, Tyr, Val which are implicated in ACE inhibition. Such peptides having ACE inhibition were isolated and they include Val-Ser-Leu-Pro-Glu-Trp f(21–26), Gly-Val-Ser-Leu-Pro-Glu-Trp f(20–26), Leu-Lys-Gly-Tyr-Gly-Gly_Val-Ser-Leu-Pro-Glu-Trp f(15–26), and Tyr-Gly-Gly_Val-Ser-Leu-Pro-Glu-Trp f(18–26) of α-La. The highest ACE inhibition was found with f(21–26) and f(15–26). All the peptides had the same C-terminal tryptophan and their molecular masses were around 1,000 Da.

Other fragments of α-La, namely, Ile-Val-Glu-Asn-Asn-Gln-Ser-Thr-Asp-Tyr-Gly f(41–51), Phe-His-Thr-Ser-Gly-Tyr-Asp-Thr-Glu-Ala f(31–40), Leu-Asp-Gln-Trp f(115–118), and Leu-Asn-Asn-Asp f(101–104) were also released during hydrolysis of α-La with thermolysin, but their ACE inhibition was not assayed. The peptides were observed to have hydrophobic amino acid residues at the C-terminal and a molecular mass of less than 3000, hence likely to show ACE activity. α-La peptides with ACE inhibitory activity also include the decapeptide Val-Gly-Ile-Asn-Tyr-Trp-Leu-Ala-His-Lys f(99–108), pentapeptide Trp-Leu-Ala-His-Lys f(104–108), and the tripeptide Tyr-Gly-Leu (f50–52), obtained with trypsin alone, and with an enzyme combination containing pepsin, trypsin, and chymotrypsin (Pihlanto-Leppälä et al., 2000), the tetrapeptide α-lactorphin Tyr-Gly-Leu-Phe f(50–53), and the dipeptides Tyr-Gly, f(18–19), Tyr-Gly f(50–51), and Leu-Phe f(52–53) (Mullally et al., 1996). Peptides may be able to bind minerals (Baumy and Brule, 1988) and have shown a higher affinity for Fe^{2+} than the native proteins. WPC hydrolysates derived with alcalase, trypsin, papain, and flavorzyme (EC 3.4.11.1) show iron solubility (Kim et al., 2007b). Peptides obtained with alcalase were short, less than 3,000 Da, and had the highest ability to bind iron (0.2 mg/kg) and gave high iron solubility of 95%. Short peptides were, as a result of a high DH, responsible for alcalase's broader specificities in cleaving various peptide bonds. The exact mechanism of iron binding to the peptides has not been established; however, their amino acid analysis showed higher concentrations of Ala, Lys, and Phe compared to others with less binding ability.

Investigations on the antioxidant activity of hydrolysates from bovine whey proteins by commercial proteases have revealed that Corolase PP, a complex mixture of enzymes that acts synergistically on whey proteins, was the most appropriate enzyme in obtaining antioxidant hydrolysates from α-La (Hernández-Ledesma et al., 2005). The hydrolysates fractionated by a 3,000 Da membrane had an oxygen radical absorbance capacity fluorescein (ORAC-FL) value of 2.315 μmol of Trolox equivalent/μg of protein. Other enzymes, pepsin, trypsin, chymotrypsin, and thermolysin released hydrolysates with lower ORAC-FL value corresponding to 0.970, 0.942, 1.755, and 1.365 μmol of Trolox equivalent/μg of protein, respectively. Differences in the radical scavenging activity among hydrolysates were attributed to differences in the size and amino acid sequence of the peptides released by the proteases.

Substrate pre-treatment

Many properties of protein substrates are known to affect enzyme hydrolysis. Substrates may be altered in order to improve their enzyme susceptibility. One such method is the formation of β-Lg/retinoic acid or β-Lg/retinol. This was found to be resistant to tryptic digestion (Shimoyamada et al., 1996).

Heat denaturation of whey proteins

Heat-denaturation of whey proteins is generally acknowledged to proceed through a two-step process: unfolding at low denaturing temperatures followed by aggregation. The individual fractions in WP differ in their thermal stability in the order Ig < BSA < β-Lg < α-La < proteose peptone. Heat-aggregated whey proteins produced better films (Floris et al., 2008) due to the SH/S-S exchange reactions (rearrangement) which occur during heat-denaturing. In addition, heat-denaturation led to increased viscosity, surface hydrophobicity and susceptibility to some enzymes while being inhibitory to others.

There exist differences in whey protein denaturation patterns depending on whether WPI, WPC, WP-casein mixtures or pure β-Lg or α-La are heated individually compared to heat-treatment of their mixtures in whey proteins (van Vliet et al., 2004). When heated alone, α-La did not form aggregates, but in a mixture with β-Lg it was incorporated into the disulphide-bonded and the hydrophobically associated aggregates as well as forming α-La dimers and other oligomers. The presence of α-La diminished the proportion of smaller

aggregates and increased the number of very large aggregates within both variant protein mixtures. In the presence of α-La, β-Lg A was converted into a series of disulphide-bonded and the hydrophobically associated aggregates more slowly, but with a greater proportion of hydrophobically associated aggregates, than β-Lg B. These patterns: are similar to that when β-Lg A or B are heated on their own. These and other results indicate that the mechanism of aggregation of α-La/β-Lg mixtures is governed by β-Lg (Schokker et al., 1999).

In the presence of β-Lg heating causes aggregation of α-La. Differences in the thermal stabilities of whey proteins were exploited by varying the composition of the liquid whey protein concentrate in terms of total protein, lactose and calcium content, and pH value. In effect, β-Lg was selectively denatured in a process which yielded pure α-La (Bramaud et al., 1997; Tolkach et al., 2005). During the heating of mixtures of α-La and β-Lg at low ionic strength, soluble aggregates form via disulphide and hydrophobic interactions. Homopolymers of each protein, as well as heteropolymers, which are formed from disulphide-bonded α-La dimers, 1:1 aggregates of β-Lg:α-La dimer and nonnative monomers and dimers etc., of both α-La and β-Lg have been observed (Havea et al., 2000; Hong and Creamer, 2002). The presence of α-LA diminished the proportion of smaller aggregates and increased the number of very large aggregates within both variant protein mixtures. Preheating β-LG decreased the extent of loss of α-LA from the mixtures, suggesting that the bond shuffling that occurs during heat treatment is enhanced by the formation of MG intermediates and by thiol catalysis (Hong & Creamer, 2002). Heat-induced interactions within mixtures of α-La and β-Lg generated large aggregates that were held together by disulphide bonds and intermediate-sized aggregates that were held together mainly by disulphide bonds and to a lesser extent by non-covalent bonding (Dalgleish et al., 1997; Havea et al., 2000).

Aggregation rate of α-La at 80°C increased an order of magnitude in the presence of β-Lg. This was attributed to an interaction between denatured α-La and β-Lg molecules. It is suggested that α-La is denatures easily but then reverts rather than to form aggregates (Rüegg et al., 1977). The renaturation reaction is not favored in the presence of denatured α-La with denatured β-Lg molecules (Hines and Foegeding, 1993). Thermal denaturation of β-Lg A, β-Lg B, and α-La in whey protein isolate solutions showed that α-La denatured more slowly than β-Lg at all temperatures. The activation energy of either β-Lg A or β-Lg B increased with increasing pressure whereas the activation energy of α-La showed a distinctive maximum at 400 MPa (Hinrichs and Rademacher, 2004). The less reactive α-La is available to aggregate with the unfolded β-Lg by disulphide-bond exchange. A critical part of the process is for the S–S bond of α-La to physically contact one of the CysH residues of another protein, generally β-Lg, and for the α-La to obtain a CysH, which could then, create new S–S bonds with α-La, α-La aggregates and α-La:β-Lg dimers. It is very likely that the critical α-La:β-Lg interactions take place in a hydrophobic environment. On increasing the pressure to 800 MPa, β-Lg was shown to be > 80% irreversibly denatured whereas 40% of α-La was denatured. This was attributed to the conformational stabilizing bonds inherent to each protein's structure (Hinrichs et al., 1996).

WPI powders were dry-heated under controlled water activity ($a_w = 0.23$) at different pH values (6.5, 4.5, and 2.5) at 100°C for up to 24 hours (Gulzar et al., 2011). Dry heating was accompanied by a loss of soluble proteins (native-like β-Lg and α-La) and the concomitant formation of aggregated structures that increased in size as the pH increased. The loss of soluble proteins was less when the pH of the WPI was 2.5; in this case only soluble aggregates were observed. At higher pH values (4.5 and 6.5), both soluble and insoluble aggregates were formed. Intermolecular disulphide bonds between aggregated proteins predominated at a lower pH (2.5), while covalent cross-links other than disulphide bonds were also formed at pH 4.5 and 6.5. This result underscored the influence of the denaturing pH in the production of different heat-denatured WPI products and hence manipulating their properties through pH-controlled aggregation. Denaturation of WPC at pH 6.6 and 7.5 influenced their solubility in a manner that was closely related to the heating conditions (Stănciuc et al., 2012). The denaturation mechanism involved a number of consecutive conformational changes in the molecules. A curvature in Arrhenius plots was observed around 75°C, unlike 90°C observed by Tolkach and Kulozik (2005) with β-Lg, indicating changes in the reaction mechanism. Furthermore, the deflection of Arrhenius plot reflected the generally accepted two-step denaturation/aggregation process of whey proteins.

Heat denaturation of pure β-Lg. Heat treatment of milk causes impaired clotting properties, which makes heated milk unsuitable for cheese production. Up to 60°C the effect of heat on β-Lg seems limited to the dimer-monomer transition (Fig. 19). However, as the temperature of a β-Lg solution (at pH 6.5) is raised to 70°C, Trp^{61} is exposed and is reversibly buried again after rapid cooling (Mills, 1976). The kinetics of denaturation of β-Lg has been extensively studied using a wide variety of conditions, e.g. in whey protein isolates, milks, milk salts, various pH's etc. When the temperature of the protein solution increases above 60°C, the β-Lg molecule undergoes a reversible partial unfolding and forms a thermally induced molten globule state (U_{MG}). Irreversible intermolecular interactions that result in the formation of polymers and aggregates (U_n, U_m).

Denaturation kinetics of β-Lg were reported to be of the of 1.5 in skim milk (Dannenberg and Kessler, 1988), about 1.4 (Kehoe et al., 2011) in pure β-Lg and β-Lg in whey proteins or first order kinetics in heated milk (Claeys et al., 2001). Higher reaction orders of 1.96 and 1.98 for β-Lg A and B, respectively were reported in a study that revealed that the presence or absence of NaCl or $CaCl_2$ altered the reaction order (Croguennec et al., 2004). Elsewhere, β-Lg denaturation in whey protein-depleted milks was shown to have a higher reaction order than in whey protein-enriched milks. When β-Lg was denatured in the presence of $CaCl_2$ at pH 7, the rate-limiting step was the aggregation step, resulting in an overall second-order reaction (Oldfield et al., 2005).

The strong dependence of β-Lg denaturation showed that after the dimer-monomer transition, the onset of denaturation/aggregation pathway is the unfolding of β-Lg and the formation of a nonnative monomer whose particle size is the same as that

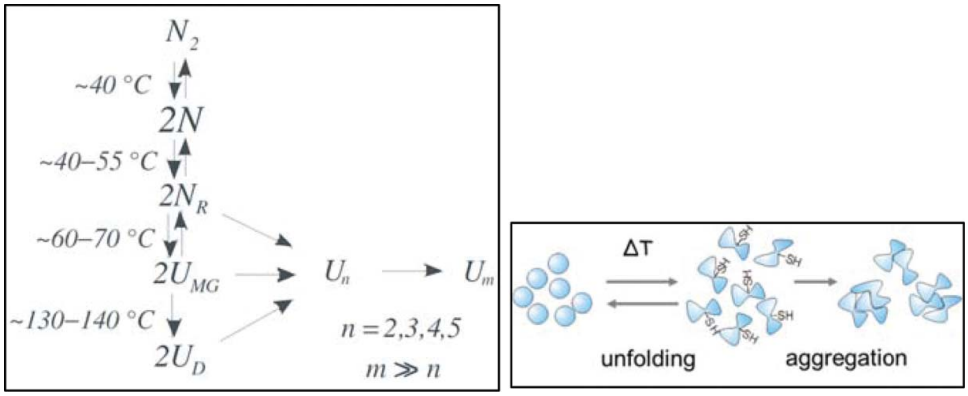

Figure 19. Mechanism of the thermal denaturation of β-Lg (adopted from Tolkach and Kulozik (2007)). N: native as dimer (N_2) or monomers (2N). U: unfolded, R: reversible state.

of the native monomers although its characteristics differs from those of the native β-Lg. The nonnative dimers and oligomers were formed presumably mainly via thiol-disulphide exchange and to a lesser extent via thiol–thiol oxidation and noncovalent interactions. In later stages aggregates with a continuous distribution of molar masses from 10^5 to 2×10^6Da were formed, presumably via the incorporation of monomers and small aggregates into larger aggregates mainly through disulphide bonding (Schokker et al., 1999). Croguennec et al. (2003) showed that the rearrangement of the disulphide bonds in β-Lg occurred causing the formation of nonnative monomeric protein (Fig. 20).

The nonnative monomers were reportedly brought about by a rearrangement of the disulphide bonds in native β-Lg leading to the sulfhydryl group on Cys^{119} being exposed and a disulphide bond-forming between Cys^{119} and Cys^{121}. As well as this another recent study has found monomeric β-Lg species where the other disulphide bond, Cys^{66}-Cys^{160}, has been cleared to leave an exposed sulfhydryl group on one of the cysteines. It has been suggested that these nonnative monomers are less reactive than unfolded β-Lg with the native disulphide bonds intact (Kehoe et al., 2011). These nonnative monomers behave differently to native β-Lg in that they are insoluble at pH 4.6 and elute slightly earlier in GPC chromatography than native β-Lg. In our recent work, it was found out that heat-denaturing of β-Lg at pH at 80 °C and pH 4.6, 6.8 and 8 led to the formation of product particle sizes that confirmed formation of nonnative monomers whose particle sizes resembled those of the undenatured protein (Leeb et al., 2011).

Furthermore, the reaction kinetics of β-Lg is greatly dependent on the temperature at which the protein is heated. There is a bend in the Arrhenius plot at ~90°C; below this temperature the rate of β-Lg unfolding limits aggregation reactions, at

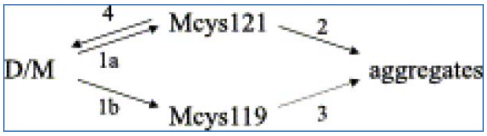

Figure 20. Scheme of the first events taking place during the heat-induced denaturation/aggregation of β-Lg. D/M: dimer–monomer equilibrium mixture at 20°C; $Mcys^{121}$, non-native β-Lg monomer with Cys^{121} exposed to the solvent reversibly formed during heat-treatment of β-Lg solutions; $Mcys^{119}$, nonnative β-Lg monomer characterized with non-native intramolecular disulfide bonds; and the Cys^{119} exposed to the solvent formed irreversibly during heat-treatment of β-Lg solutions; aggregates (dimer, trimer, …).

higher temperatures the β-Lg rapidly unfolds and the aggregation step is rate limiting (Tolkach and Kulozik, 2007). It was found out that whey protein denaturation causes a very slight reduction of enzyme activity during cheese-making. It is known that does not; affect the start of flocculation but has a clear effect on the clotting process (Vasbinder et al., 2003).

With the help of differential scanning calorimetry (DSC), it was shown that heat treatment of β-Lg at 80°C for 20 min resulted in complete destruction of the protein tertiary structure which increased the susceptibility of the β-Lg fraction to enzymatic hydrolysis by pepsin and trypsin (Mullally et al., 1998). DSC thermograms showed that the β-Lg fraction had higher thermal/structural stability at pH 3.0 than at pH 8.0. In addition, holding β-Lg at 50°C for 120 min at pH 3.0 or pH 8.0 decreased the structural stability of the protein.

Heat denaturation of pure a-La. Heat denaturing of α-La did not affect BAMLET formation in a complex with oleic acid (Liškova et al., 2010). In a study on the effects of temperature, cations, and pH on the progress of α-La aggregation, storage temperature was a critical factor for protein aggregation in model systems, and the speed of protein aggregate formation increased with increases in storage temperature. In addition, calcium significantly improved the thermal stability of α-La and slowed down the formation of protein aggregates (Liu et al., 2011). Heat denaturing of holo bovine α-La at 80°C in pH 7 phosphate buffer improves the surface activity of the protein at the air–water interface. Shuffling of the disulphide bridges was concluded to favor the formation of nonnative monomeric forms (Wijesinha-Bettoni et al., 2007).

When α-La is heated in solution, it can repeatedly undergo thermal transition. This transition is at a lower temperature (< ~66°C) than that of β-LG (~ 73°C) (Rüegg et al., 1977). The thermal transition temperature of the Ca^{2+}-free apo-α-La decreases to about 35°C compared to that of Ca^{2+}-binding holo- α-La of 66°C (Relkin and Mulvihill, 1996). Extensive heating (100°C for at least 10 min) in the absence of extra calcium gives rise to disulphide-bonded α-La dimers and trimers as well as some nonnative monomeric protein (Chaplin and Lyster, 1986). It is generally accepted that α-La does not polymerize by itself when heated above 70°C. Heat treatment of α-La has very little effect on aggregation and disulphide-bond interchange, partly because no free thiol is available (Considine et al., 2007). Over a temperature range of 78–94°C, the

concentration of the residual immunoreactive protein showed that denaturation of α-La was in the order of reaction of 1.5. In addition, α-La was more heat-sensitive when treated in milk than in phosphate buffer and denatured more rapidly in the apo-form than in the Ca^{2+}-saturated holo-form while thermal stability of apo- α-La decreased with the binding of oleic acid (Wehbi et al., 2005).

Impact of heat denaturation on hydrolysis. Hydrolysis of heat-denatured whey proteins showed an impact on protein susceptibility to enzyme attack. For example, heat-denatured WPI was hydrolyzed with trypsin, α-chymotrypsin, alcalase or neutrase. The hydrolysates showed an increase in the surface hydrophobicity with increasing hydrolysis. After UF, the solubility of both the permeate and retentate increased. Emulsifying activity index of the retentates was higher than that of the hydrolysates while permeates formed stable foams but hydrolysates and retentates showed poor foaming characteristics. Furthermore, the specificity of the enzyme, and DH influenced the functional properties of the peptides with trypsin hydrolysates having higher solubility, emulsifying properties and foaming properties (Mutilangi et al., 1996).

Heat-denaturing of proteins before hydrolysis is recognized as an important manipulation to improve enzyme attack. Schmidt and van Markwijk (1993) found that when purified samples of unheated α-La and of β-Lg A and β-Lg B were hydrolyzed by pepsin under pH 2.5 at 40°C, only α-La was hydrolyzed by pepsin but not by papain. After heat-denaturation for 8 min at 82°C, however, no significant effects were noticed with either enzyme on α-La hydrolysis. Furthermore, native β-Lg was not hydrolyzed by pepsin while denatured β-Lg was hydrolyzed. The researchers found that native β-Lg was rapidly hydrolyzed by papain in the order β-Lg A > β-Lg B, a property which was similar to that observed with trypsin hydrolysis (Creamer et al., 2004b). The enzyme susceptibility of the denatured protein was attributed to unfolding of the polypeptide chain.

Native β-Lg was reported to be resistant to pepsin (Schmidt and Poll, 1991). After heat-denaturing 90–100°C for 5 or 10 min, however, Guo et al. (1995) found a dramatic increase in peptic and tryptic hydrolysis. Whey proteins treated with porcine stomach pepsin, bovine chymosin, human uropepsin, and gastric juice from rat stomach at various pH values showed that although α-La and BSA were hydrolyzed, β-Lg was not hydrolyzed by pepsin under at any pH in vitro. However, heat-denatured whey proteins, including heated β-Lg, were easily hydrolyzed by pepsin. In addition, native β-Lg injected into the stomach of a rat was not digested in vivo, whereas heat-denatured β-Lg was digested in the stomach of a rat (Kitabatake and Kinekawa, 1998).

Hydrolysis of heat-denatured whey proteins is a common practice in infant formular production. Enzyme inhibition was detected during hydrolysis of heat-denatured whey proteins (Cheison et al., 2007a). Heat denaturing is most commonly used to produce extensively hydrolyzed food formulae from whey proteins because it leads to the removal of conformational epitopes although it also exposed some hidden antibody binding sites (Davis and Williams, 1998). In addition, thermal precipitation of heat-denatured β-Lg reduces the amount of residual intact protein in the preparation, which would otherwise elicit some allergenic response. However, our results show that the denatured WPI was much less easier to hydrolyze than the undenatured WPI.

Iametti et al. (2002) suggest that heat denaturing and aggregation of β-Lg upon heat treatment may hide putative sites of attack from the proteinase, leaving some regions of the protein intact. WPI denatured at 80°C for 30 min and hydrolyzed using a proteinase from *Bacillus licheniformis* showed that gelation was found to take place at a constant DH, independent of the enzyme concentration, while the DH decreased with increasing temperature unlike in the undenatured samples (Ipsen et al., 2000b). Hence simultaneous hydrolysis and thermal treatment results in gels with a microstructure dependent on the temperature (Ipsen et al., 2000a). Heat-denaturation exposes hydrophobic core in the protein, may lead to aggregation as well as masking potentially allergenic epitopes in the parent protein. It may, therefore, be used to pre-treat the protein in order to modify its properties, molecular structure and hence enzyme attack sequences.

One factor which has remained ignored in the heat-denaturation processes is the environment under which whey proteins were denatured prior to hydrolysis. For example, Mutilangi et al. (1995) treated WPI at 85°C and pH 4.6. At this pH, α-La is closer to its pI while β-Lg is just below its pI and according to Pessen et al. (1985) (Fig. 3) the protein exists in octamers. In that molecular state, enzyme penetration is hindered which likely leads to less accessibility to β-Lg, hence reduced hydrolysis. Heat-denaturing at the physiological pH of milk (pH 6.6) by Croguennec et al. (2004) showed the formation of nonnative monomers due to unfolding. Thus, native β-Lg molecules unfolded to native thiol-exposed monomers and nonnative thiol-exposed monomers. It was also observed that whereas the unfolded native monomers formed intermediate oligomers (dimer, trimer, tetramer) which rapidly combined to larger aggregates in the presence of $CaCl_2$ and to a lesser extend in the presence of NaCl, the nonnative monomers trapped β-Lg molecules and slowed down its aggregating behavior at pH 6.6.

The environment pH is critical during denaturation because there was formation of reversible formation of non-native monomer with exposed Cys^{121} (Mcys121) and a stable nonnative monomer with exposed Cys^{119} (Mcys119) which exhibited both a larger hydrodynamic conformation than native monomer and low solubility at pH 4.7 (Croguennec et al., 2003). The formation of monomeric species throughout heat-induced denaturation of native β-Lg monomers is faster than their subsequent aggregation. Heating at neutral pH was enhances emulsification and foaming while heating at an acidic pH results in high viscosity, lower gel temperature and high water absorption.

Extended exposure to temperatures >60°C can irreversibly affect the solubility of whey proteins (de Wit, 2009) and change the relative hydrophobicity at the protein surface leading to exposure of nonpolar hydrophobic residues thereby increasing hydrophobic attraction. Hydrophobic interactions are reported to increase with increasing temperature up to ~70°C, after which they diminish. Exposure of sulfhydryl groups and hydrophobic patches, due to unfolding, decreases protein stability and solubility. After heating WPI adjusted to pH 6.4 at various

temperatures for different holding times like 60°C for 15 min and at 65°C, 70°C, 75°C and for either 5 or 15 min as well as at 80°C for 10 min, O'Loughlin et al. (2012) hydrolyzed with Corolase PP (an enzyme with chymotrypsin, elastase, tryptic, aminopeptidase and along with carboxypeptidase A1, A2, and B exopeptidase activities) to a fixed DH of 5%. They found out that heating led to large aggregate formation, higher viscosity and surface hydrophobicity while increasing the hydrolysis speed more than with the unheated WPI. The improved hydrolysis was particularly detected after aggregation of WPI by thermal pretreatment at ≥75°C for 5 min.

Evidently the effect of protein composition for example WPI, WPC, pure β-Lg, pure α-La or presence of salt and/or lactose play a crucial role in the product characteristics after heat-denaturation. In addition, the heat-denaturation pH, temperature, holding time, and buffer influence the SH/S-S exchange in the protein resulting in differences in protein aggregate as well as gel formation. These are variables which, in addition to protein concentration, must be considered during heat-denaturation of whey protein fractions. To date, these variables have received little consideration and present potential challenges in the interpretation of the results.

Glyco-conjugation

Chemical modification by succinylation or acetylation of the ε-amino group, amidation, esterification, phosphorylation and thiolation are protein modifications reported in the literature. Maillard reaction is a popular method used to improve protein functionality without altering its nutritional properties although it was reported that chemical modification altered nutritional value and also increased the resistance of adjacent peptide bonds to peptic and tryptic hydrolysis meaning that it can be used as a means to steer the enzyme hydrolysis process.

Maillard reaction causes changes in flavor and structure due to protein-reducing sugar interactions during food processing resulting in the formation of protein-sugar-conjugates. It is these conjugates that show new properties with multifunctional application possibilities, including increased surfactant properties (Nagasawa et al., 1996; Akhtar and Dickinson, 2003; Akhtar and Dickinson, 2007; ter Haar et al., 2011b), anti-oxidant properties (Jing and Kitts, 2004; Yilmaz and Toledo, 2005; Chawla et al., 2009; Gu et al., 2010) and radical scavenging properties (Sumaya-Martinez et al., 2005). In addition, glycosylated proteins possessed anti-bacterial effect against pathogenic bacterial strains (Adamberg et al., 2005). Regarding allergenicity and immunogenicity, anti-β-Lg antibody was markedly reduced after immunization of BALB/c, C57BL/6, and C3H/He mice with acidic oligosaccharide-protein conjugates (Hattori et al., 2004). This was also observed with protein engineered glycol-conjugates (Tatsumi et al., 2012) in a study which concluded that site-specific glycosylation is an effective method to reduce the immunogenicity of β-Lg while masking of epitopes by high-mannose chains provides an effective means with potential to be used in the reduction of protein immunogenicity. Indeed, it was confirmed in a recent study that conjugating WPI with maltose was an effective way to reduce the antigenicity of α-La and β-Lg (Li et al., 2011). Conversely, reaction with galactose, tagatose, and dextran of 10 or 20 kDa impaired β-Lg proteolysis and, consequently, increased the IgG- and IgE-reactivities of hydrolysates, regardless of the carbohydrate used (Corzo-Martínez et al., 2010).

Prominently, this nonenzymatic conjugation of α-amino groups or amino side chain of lysyl groups with reducing sugars involves a complex reaction leading to the formation of melanoidine-like brownish complexes. The initial stages are characterized by the formation of the Amadori products via the Schiffs base. In the subsequent advanced stages, the Amadori product breaks down into a variety of different compounds which will form melanoidins in the final stages of the reaction (van Boekel, 1998).

Various factors influence protein glycosylation including a_w, temperature, pH and buffer, type of sugar, molar ratio of sugar to protein, protein properties and reaction temperature and time. Essentially, a_w affects the Maillard reaction through concentration and diffusion. With decreasing a_w, the concentration of all involved reactants is increased, which to a certain degree favors the glycosylation reaction until, when at extremely high concentration diffusion becomes more difficult and slows the reaction down (van Boekel, 2001). Heating dry mixtures of lactose and caseinate at 60°C, Ge Pan and Melton (2007) found maxima of the Maillard reaction (furosine level) at 67% relative humidity after 12 and 24 hours, whereas at 50% relative humidity (RH) this was reached after 48 and 96 hours. However, Maillard reaction kinetics in milk powder (as loss of lysine), showed no statistically significant maximum at 50°C and 60°C, at intermediate a_w (Pereyra Gonzales et al., 2010). Nevertheless at a_w >0.7 a dilution effect could be observed whereas at low aw (0.33) the reaction rate was reduced at low temperatures (37°C) due to the high viscosity of the system.

The reactivity of sugars varies depending on the level of mutarotation and the carbohydrate size. Sugars with higher concentrations in the open chain form promote browning reaction to a higher degree (van Boekel, 2001). Jing and Kitts (2004) found a higher reactivity of ribose (a pentose) as compared to glucose (an aldose), which was reported to be more reactive than fructose (a ketose). The higher reactivity of pentoses compared to hexoses was confirmed by Laroque et al. (2008) in shrimp hydrolysates who found that xylose was more reactive than ribose, whereas no significant difference was found between glucose and fructose. In a study on the kinetics of the loss of lysine in casein with different sugars by Naranjo et al. (1998) the reactivity of the four different sugars was reported in the order glucose < lactose = maltose < fructose. It was argued that the difference in reactivity of fructose could be explained by a different mechanism of interconversion of anomers. In case of fructose a complex reaction of tautomerization takes place whereas in the other three sugars the underlying mechanism is mutarotation. Similar results were obtained for sugars of high molecular weights such as dextran. In reaction with whey proteins, dextran of lower molecular weight (10 kDa) proved to be more reactive than dextran of a higher molecular weight (20 kDa) (Jiménez-Castaño et al., 2007). Choi et al. (2005) assumed that steric hindrance of high molecular dextran (10 kDa) limited the protein-dextran conjugate ratio.

The reactivity of sugars, therefore, seems to decrease with increasing sugar size and molecular weight. Moreover, the type of sugar does not only influence the reaction rate, but the properties of the resulting glycoproteins are influenced by the type

of sugar as well (Chevalier et al., 2002). Sun et al. (2006) analyzed the effect of sugar type and time on the glycosylation degree (DG) of α-La and found out that D-allose, a pentose, was more reactive compared to the hexoses; glucose and fructose while fructose exhibited less reactivity than glucose. The blocking of lysine increased with heating time in all cases but the DG depended on the sugar type.

The susceptibility to glycosylation depends on the protein. With respect to the glycosylation reaction, α-La contains 13 potentially reactive groups; 12 lysyl residues and one terminal amino group (Sun et al., 2006) and, therefore, it can be assumed that for each molecule of α-La at least 13 possible reactive groups exist (Fig. 14). Protein β-Lg on the other hand has 16 potential glycosylation sites (15 lysyl residues and a terminal amino acid Ile) (Fig. 13). The two major whey proteins differ in their glycosylation speeds (Nacka et al., 1998). Moreover, the susceptibility of proteins to glycosylation is affected by its primary structure. The reactivity of the lysyl residues was influenced by vicinal amino acids and their nature (Morgan et al., 1997; Mennella et al., 2006). Nacka et al. (1998) analyzed the effects of glycosylation on β-Lg and compared it to the glycosylation rate of α-La. It was found that the DG and extend of α-La glycosylation exceeded that of β-Lg. Lund et al. (2005) investigated the kinetics of lactosylation of α-La in an aqueous system. Not all lysyl residues are involved in the reaction but some residues are reported to be the main targets for glycosylation. Siciliano et al. (1999) investigated the structural modifications in commercial milk products and found that the main modification site in α-La was Lys^{98}. Marvin et al. (2002) assessed the whey protein quality in infant formula and found lactosylated α-La with modifications at residues Lys^{5}, Lys^{13}, Lys^{94}, Lys^{108}, and Lys^{122}. This indicates differences in the reactivity of the lysyl residues towards glycosylation. In β-Lg, glycosylation was probed using tryptic hydrolysis and it was shown that glycosylation of Lys^{69} was slower than Lys^{70} (Chevalier et al., 2001).

During glycosylation of β-Lg with arabinose, galactose, glucose, lactose, rhamnose and ribose at 60°C, reaction was detected at Lys^{14} and Lys^{47} were modified in the presence of galactose, glucose or lactose. Lys^{69}, Lys^{75}, and Lys^{135} were modified only in the case of protein glycated with glucose. Lys^{100} was modified only when protein was glycosylated with lactose. When acidic oligosaccharides alginic acid oligosaccharide and phosphoryl oligosaccharides were used, Lys^{60}, Lys^{77}, Lys^{100}, Lys^{138}, and Lys^{141} were glycosylated (Hattori et al., 2004). The pentoses reacted rapidly with β-Lg and it was not possible to identify the glycosylation patterns. Evidently, the rate of protein glycosylation is dependent on the sugar size and saccharide type and decreased when the saccharide size increased (ter Haar et al., 2011a). This trend was also reported with casein glycosylation kinetics (Kostyra et al., 2010), whereby kinetics of glycation depends not only on the structure of protein but also on the structure of saccharide.

As long as no equilibrium is reached, the products of any chemical reaction will increase with time. According to the Arrhenius equation, the rate constant increases with temperature which elevates the browning rate. This correlation applies to both the early and final stages of the Maillard reaction. For instance, the formation of the Amodori product in the reaction between dextran and β-Lg increases with rising temperature (range of 50°C–60°C) (Naranjo et al., 1998; Jiménez-Castaño et al., 2005; Pereyra Gonzales et al., 2010).

Although a lot of reports on nonenzymatic glycosylation of whey proteins in general and β-Lg in particular exist, there is a paucity of reports on the glycosylation of α-La. The parameters affecting the reaction are most likely the same in all glycosylation reactions but little information exists on their interdependency as well as predictive kinetics. Enomoto et al. (2009) performed glycosylation experiments of α-La with subsequent phosphorylation. It was shown that glycosylation with maltopentose and subsequent phosphorylation affected the physiological functions of α-La. The apoptotic activity of α-La was reduced, the formation of pro-inflammatory cytokines was suppressed, calcium phosphate-solubilizing ability of α-La was enhanced, a decline in antibody response and a decrease in denaturation temperature of α-La were reported.

Reaction orders were reported to differ for α-La glycosylation. First-order kinetics were used by Pereyra Gonzales et al. (2010) and Naranjo et al. (1998). The calculated activation energy for lactose (Naranjo et al., 1998) in the case of casein was determined as 125±4.2 kJ/mol while that for milk powder varied from 121.1 kJ/mol (for $a_w = 0.43$) to 135.4 kJ/mol (for $a_w = 0.85$) (Pereyra Gonzales et al., 2010). Conversely, the formation of the Schiff base was characterized using second order kinetics by Lund et al. (2005). The rate constants increased with temperature, which dependence was analyzed according to the Arrhenius equation. The activation energies for the protonated and unprotonated α-La were 161 ± 49 and 34 ± 2 kJ/mol (pH 6.0–7.9, respectively, at 50–60°C and in an aqueous solution with ionic strength of 0.080M (NaCl). Obviously the pH affected the lactosylation of α-La because of the differences in the activation energy for the protonated and the unprotonated α-La. Kinetics for temperatures below 60°C could be determined with sufficient accuracy, because above this temperature the appearance of a denatured form of α-La, the molten globule state (MGS), was likely and that the denaturation effects might affect the reaction kinetics (Rasmussen and Bjerrum, 2003; Lund et al., 2005). The glycosylation of proteins obviously has an effect on digestibility and bioactivity. By using the decreased digestibility new prebiotics could be created. These conditions, however, do not limit Maillard reaction under the promising new frontier of gamma-irradiation as reported by Chawla et al. (2009).

These reports indicate that the composition of the protein substrate, glycosylation temperature and pH as well as time play a crucial role. Besides, the sugar type and size determines the glycosylation rate as well as the extent to which the Maillard reaction may proceed. Protein hydrolysis patterns were altered with resistance detected for trypsin with glycosylated whey proteins (Adamberg et al., 2005) as well as trypsin and Lys-C in human serum albumin, HAS (Lapolla et al., 2004). It is clear that lysine loss has deleterious effects on the nutritional quality of infant formula (Alonso and Zapico, 1994). There is still controversy on whether Maillard reaction products are a health hazard or health-promoting (Meltretter and Pischetsrieder, 2008).

Nevertheless, a clear distinction ought to be made between the early and late stages of glycosylation with the products of the latter being seemingly harmful to health. Since the enzyme attack patterns are dramatically altered after glycosylation, and since trypsin specificity limits attack to lysyl and arginyl residues, it is evident that glycosylation at lysyl residues has potential to alter trypsinolytic patterns in proteins with the result that a different peptide composition may be obtained. Glycosylation, therefore, offers real opportunities for peptide tailoring from protein sources of nutritional and biofunctional interest in the food and pharmaceutical industry. There is still a huge potential in the development of analytical tools for the elucidation of glycosylation products, especially those based on mass spectrometry (Meltretter and Pischetsrieder, 2008; Oliver, 2011).

General conclusions from our work

The choices of enzymes to be used in protein hydrolysis as well as the objectives of protein hydrolysis have hitherto been motivated by the speed of reaction and the end products. By hydrolysis at trypsin and chymotrypsin optimum, the enzymes attacked the proteins randomly and simultaneously. In the process, trypsin released a number of peptides at the same time from β-Lg (Fig. 21). During hydrolysis of whey proteins, experiments at optimum conditions showed no remarkable differences in their attacks on either α-La or β-Lg (Cheison et al., 2011d). The two proteins were attacked almost simultaneously. Hydrolysis at lower temperature and low pH diminished the enzyme attack on the proteins while the release of peptides, apart from being limited, was sequential and ordered. The results from this region revealed that trypsin attacked the N- and C-terminals of β-Lg (Lys^{8}-Gly^{9}, Lys^{100}-Lys^{101}, Arg^{124}-Thr^{125}, Lys^{141}-Ala^{142}, and Arg^{148}-Leu^{149}) (Cheison et al., 2010; Cheison et al., 2011a; Cheison et al., 2011b) almost immediately upon addition while the protein centre remained intact. Hydrolysis of whey proteins was poor, with limited selectivity for either of the main proteins (Cheison et al., 2011d; Lisak et al., 2013). A switch to higher temperature and high pH led to an indiscriminate depletion of the proteins with little or no selectivity. In addition, the hydrolysis process was rapid at the beginning and declined shortly thereafter. This region showed that controlled hydrolysis of the proteins was not possible. With β-Lg, the enzyme released a large number of nonspecific peptides while the protein core was easily accessible and hydrolyzed by trypsin (Cheison et al., 2010; Cheison et al., 2011a; Cheison et al., 2011b).

At pH 7.8 temperatures above the optimum, i.e. 40–50°C, trypsinolysis followed a mixed specific and non-specific pattern. Notably, at higher temperatures, trypsin behaved like chymotrypsin with the result that peptide bonds formed by bulky, hydrophobic amino acids were cleaved. This was postulated to be due to the steric 'stretching" of the catalytic pocket in trypsin, which enabled the large amino acids to interact with the catalytic triad. The

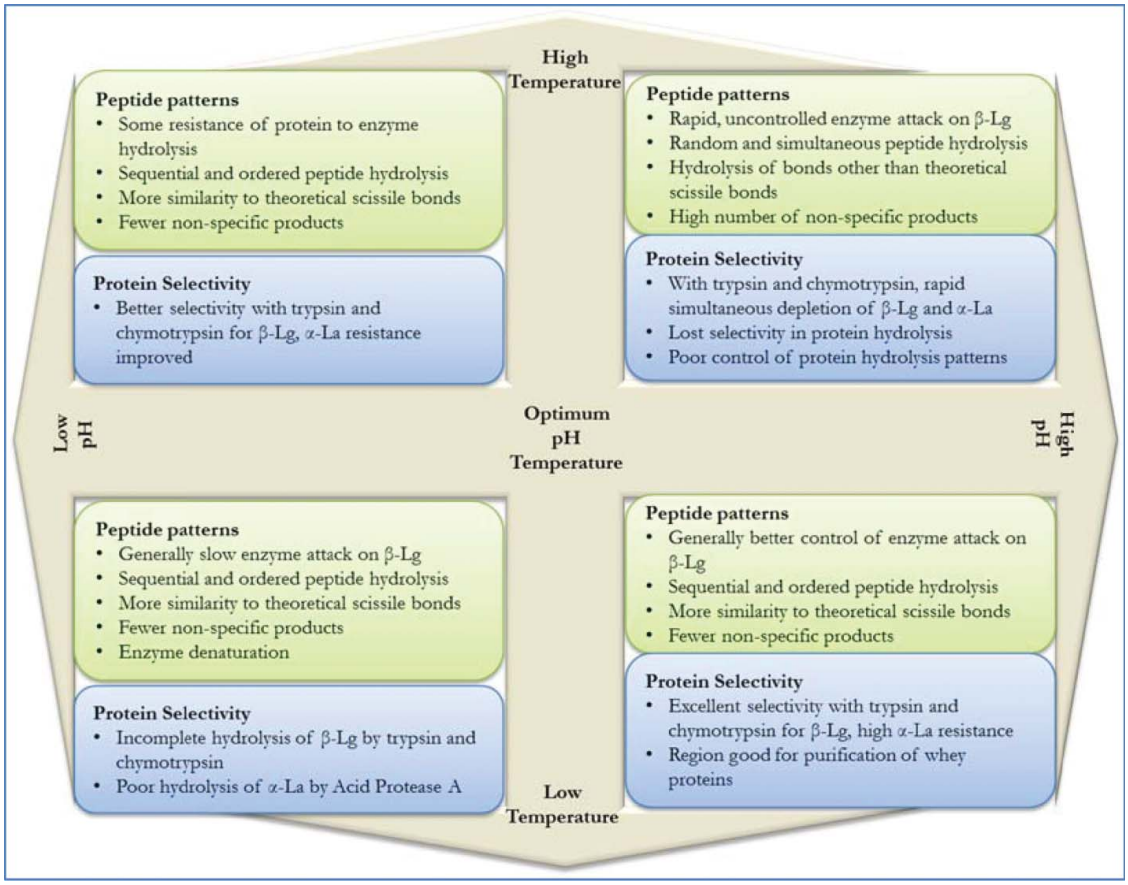

Figure 21. Summary and conclusions showing effects of temperature-pH combinations on both peptide release in β-Lg and whey protein hydrolysis (adopted from (Cheison, 2013)).

deviation was also noted for higher DHs than 1% and it remains in our interest to establish the borderline DH beyond which trypsin behaves otherwise.

Monitoring of the peptide evolution under different buffers (Cheison et al., 2011c) against time revealed a simultaneous release of peptides f(1–8), f(15–40), f(41–60), and f(142–148) from the onset of hydrolysis. In addition, nonspecific chymotrypsin-like activity leading to the cleavage of Tyr^{20}-Ser^{21}, a feature which was reported in many studies working with pure-TPCK-treated trypsin, was observed to occur randomly at the initial stages of trypsinolysis. This might call for a redefinition of trypsin specificity to include this unique peptide bond. Trypsinolysis shifted from the C-terminal of lysine formed peptides to the N-terminal. Increasing potassium-phosphate buffer likely led to dimerization and hence resistance of a core region of β-Lg. Cleavage of arginyl residues was generally faster than that for lysyl residues throughout. Typically, nonspecific cleavage was also influenced by the hydrolysis milieu, being detected at higher (50°C) rather than at lower (25 or 37.5°C) temperatures. Peptide resistance to trypsinolysis was detected at 50°C and pH 8.65 for the bond Lys^{91}-Val^{92} and Lys^{138}-Ala^{139} and at pH 9.5 for Lys^{70}-Ile^{71}.

Hydrolysis under lower pH values than the neutral pH (acid pH region) revealed that time influenced the peptide types which were released (Cheison et al., 2011b). Thus, although it took longer at pH 4 to release peptides from β-Lg, for example, over time the patterns were detected. In contrast, the core domain was relatively resistant to trypsinolysis, especially at low pH and high substrate concentration. Arg^{40}–Val^{41}, Lys^{141}–Ala^{142}, and Arg^{148}–Leu^{149} were generally attacked initially, followed by Lys^{8}–Gly^{9} and Lys^{138}–Ala^{139}. The most resistant bonds were Lys^{60}–Trp^{61}, Arg^{124}–Thr^{125}, and Lys^{135}–Phe^{136} as well as the neighbor lysines Lys^{70}–Lys^{71} and Lys^{100}–Lys^{101} as well as Lys^{77}–Ile^{78}, especially at low pH. There are opportunities to change the resistance of different cleavage sites of β-Lg with changes in the hydrolysis milieu in order to either produce desired peptides or protect some cleavage sites hence preserve some precursor peptides with target bio-functional properties.

Interesting results were witnessed during hydrolysis of whey protein by both trypsin (Cheison et al., 2011d) and chymotrypsin (Lisak et al., 2013) at a combination of low temperature (25°C) and high pH (8.5). At these conditions, the pH induced a dimer-to-monomer dissociation in β-Lg leading to better enzyme penetration and β-Lg depletion. However, α-La was resistant to both trypsin and chymotrypsin and this combination offered great potential for selective enzyme hydrolysis of β-Lg with potential to purify α-La. Hydrolysis of β-Lg depended on the genetic variants, with β-Lg A being generally more susceptible to trypsin hydrolysis than β-Lg B. The dependence of the trypsin hydrolysis temperature and pH on the resistance of α-La indicates the influence of the Tanford transition (Tanford et al., 1959) conditions (>40°C and pH>7.5) on the dimer-monomer transition, increased hydration and enzyme penetration and hence improved hydrolysis of b-Lg. Higher resistance to trypsin by a-La was detected at <30°C whereas less resistance was detected at >40°C. The highest possible recovery of native a-La (z68.0%) was achieved at 25°C and pH 8.5. Under these conditions, trypsin was operating under a "temperature stress," while, at pH 8.5 the dimer-to-monomer dissociation of β-Lg was promoted. The lowest recovery of pure a-La (8.0%) was at obtained 50°C and pH 8.5.

Hydrolysis of whey proteins at acidic pH showed that Acid Protease A could deplete α-La in a procedure which showed high resistance of β-Lg (Cheison et al., 2012). The method offered potential for the purification of β-Lg with membranes. Resistance of a-La was higher at low pH near the enzyme optimum of pH 2.5, which was reversed as the pH increased. Temperature lower than the enzyme optimum was favorable for selectivity because at 45°C there was aggregation/gelation while selectivity at 30°C was lower than at 37.5°C.

In summary, our results show that potential for the steering of the enzyme toward the cleavage and/or avoidance of specific bonds in a protein using the hydrolysis environment, which introduces a new concept in "peptide design." In this way, there exists the possibility to influence the enzyme attack pattern and hence peptide compositions through the choice of reaction conditions away from the enzyme optimum pH, temperature, and possibly also the ionic strength. Further, the benefits include the possibility of determining when to stop the process if desirable peptides are already produced in order to protect them from further cleavage by a careful choice of the milieu conditions.

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