

Influence of temperature and degree of hydrolysis on the peptide composition of trypsin hydrolysates of β -lactoglobulin: Analysis by LC–ESI-TOF/MS

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ABSTRACT

Enzymatic hydrolysis of proteins is influenced, either positively or negatively, by the hydrolysis conditions, temperature, enzyme concentration and pH, as well as substrate pre-treatments, e.g. heat-denaturing, glyco-conjugation and/or cross-linking. Purified bovine β -lactoglobulin (96.0% nitrogen) was hydrolysed using trypsin (EC 3.4.21.4, bovine pancreas) at between 30 and 50 °C to degrees of hydrolysis (DHs) between 1 and ~9.0%. The time taken to reach the desired DH varied greatly, being shortest at 45 and 50 °C and longest at 30 °C. The hydrolysates were analysed by tandem liquid chromatography–electrospray ionisation time-of-flight mass spectra (LC–ESI-TOF/MS) and results showed that the detectable peptides, at both 30 °C and 35 °C, were similar at DH 1%. However, not only were the detectable peptides produced at 40–50 °C different from those produced at lower temperatures, but the trypsin released peptides due to non-specific hydrolysis of β -Lg. The pattern resembled a shift of trypsinolysis towards chymotrypsinolysis, probably due to steric ‘stretching’ and increase of the catalytic pocket, thus allowing bulky amino acids to be processed. Hydrolysis at 30 °C to DH 5% and 10% also led to the release of peptides due to non-specific cleavage by trypsin. These results indicate that trypsin could only release peptides in a predictable manner at temperatures near, but lower than, the declared optimum of 37 °C. Above this temperature and above DH 5–10% at 30 °C, hydrolysis followed a mixed trypsin- and chymotrypsin-like activity. Lys–Pro, Lys–Ile(–Pro) and Lys–Phe bonds remained stable to trypsin at all temperatures. Some peptides with a high content of hydrophobic amino acids were undetected by ESI-TOF/MS, probably due to their poor ionisation.

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1. Introduction

The hydrolysis of proteins produces peptides with improved functional, immunological and bioactive properties superior to those of the native protein. Many health effects have been found and reported in bioactive peptides. For example, blood pressure-lowering ability, cholesterol-lowering effects, antioxidant activities and enhancement of mineral absorption (Hartmann & Meisel, 2007). The bioactive peptides are used in products such as sport-drinks, infant- and hypoallergenic-foods to provide value-addition and enhance consumer health.

Many peptides, of animal protein origin, with bioactive potential have been discovered. Most of them were isolated from milk-based products or produced from milk proteins, mainly caseins and whey proteins (Hartmann & Meisel, 2007). Milk proteins consist of caseins (78.3%), whey proteins (19%) and miscellaneous proteins (2.7%). Whey proteins, which include serum albumin, immunoglobulins, α -lactalbumin and β -lactoglobulin (β -Lg), are more reactive because they dissolve in the serum. β -Lg, the major protein in whey, is responsible for most of the bioactive properties of whey proteins (Hernández-Ledesma, Recio, & Amigo, 2008). Due to their emulsifying, interfacial and gelation properties, tryptic peptides from whey proteins are of great interest to the food industry (Mota et al., 2006).

β -Lg is a small, soluble protein with a globular structure and a monomer molecular weight of 18.3 kDa. It belongs to the lipocalin family and has the ability to bind and transport small molecules, e.g. fatty acids and vitamin A, within a hydrophobic core (Ham-

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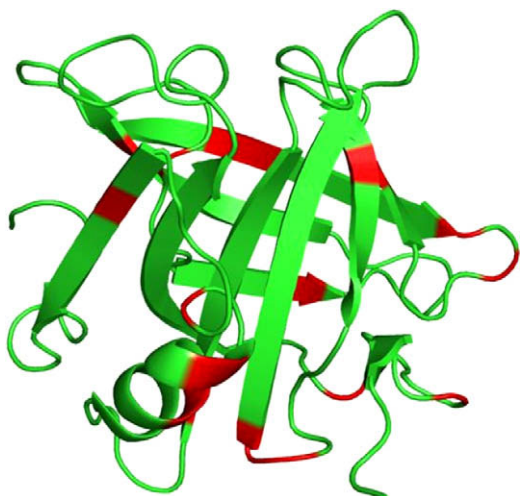


Fig. 1. Molecular structure of β -lactoglobulin with preferential cleavage sites of trypsin highlighted in red. (Compiled on <http://polyview.cchmc.org> on December 4th 2008). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bling, McAlpine, & Sawyer, 1992). β -Lg is the major whey protein in the milk of ruminants and represents approximately 10% of the total milk protein and 53% of the whey protein (Walstra, Wouters, & Geurts, 2006). It is not found in the milk of humans (Hambling et al., 1992). The primary structure consists of 162 amino acids, of which 15 are lysine and three are arginine residues. These amino acids form peptide bonds that are preferential cleavage sites for the enzyme trypsin, and are highlighted in Fig. 1.

An important task in the production of functional foods containing bioactive peptides is the production of those peptides during food processing or by deliberate enzyme hydrolysis of proteins. To make that possible it is important to know how hydrolysis is influenced by different parameters, e.g. hydrolysis pH and temperature. Hydrolysis releases free carboxyl and free amino groups which are more or less ionised, depending on the pH of the reaction. At a pH value of 7.5, at which the experiments of this work took place, the amino group is half protonated while the carboxyl group is fully dissociated. If left uncontrolled during hydrolysis at alkaline pH, the pH would decrease, leading to enzyme denaturing. Keeping the pH constant (or static, hence pH-stat) is possible by the addition of a known amount of base, e.g. NaOH, of a known concentration. The relationship between the volume of base consumed and the peptide bonds cleaved during hydrolysis is proportional. This proportionality is utilised to calculate the degree of hydrolysis (DH) by the pH-stat method. The DH is defined as the percentage of peptide bonds cleaved (h) compared to the total available peptide bonds (h_{tot}) in a protein substrate (Eq. (1)). It is this relationship which most closely reflects the catalytic action of the enzyme (Adler-Nissen, 1986).

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

Using Eq. (1), it is possible to follow the DH during the enzyme reaction. The DH is calculated directly from the consumption of base during the hydrolytic degradation, which is the principle of the pH-stat technique, according to Eq. (2).

$$\text{DH} = \frac{V_B \cdot N_B \cdot 100\%}{\alpha \cdot \text{MP} \cdot h_{\text{tot}}} \quad (2)$$

where, V_B , base consumption in ml; N_B , normality of the base; α average degree of dissociation of the -NH groups; MP, mass of protein in g; h , the hydrolysis equivalents in meqv/g protein; h_{tot} , total number of peptide bonds in the protein substrate (7.2 meqv/g of protein in this work)

For our work, we calculated the value of h_{tot} to be 7.20 for β -Lg, which is probably the first time it has ever been reported in the literature.

β -Lg exists as a dimer at physiological pH and temperature values (Hambling et al., 1992). A disulfide bond near the C-terminus (Cys₆₆–Cys₁₆₀) and one in the interior of the molecule (Cys₁₀₆–Cys₁₁₉) stabilize the compact globular conformation. When exposed to higher temperatures or pH values (above 8), the dimer dissociates. Below pH 5.5, β -Lg associates to particles of up to eight units (Fig. 2). At a pH below 3.5 no association occurs (Walstra et al., 2006). These properties are useful for both the manufacture and during the hydrolysis of β -Lg because they influence its solubility, and hence enzyme penetration and hydrolysis.

Bovine trypsin (EC 3.4.21.4) is a serine protease with a molecular weight of 23.3 kDa and consisting of 226 amino acids. Due to its nucleophilic active site, with an aspartic acid 189 sitting in a specificity pocket, the enzyme cleaves predominantly at the positively charged side chains of arginine-X and lysine-X, with X being any amino acid except a proline (Olsen, Ong, & Mann, 2004). As the lysine residue in β -Lg at position 47 is followed by a proline residue, this peptide bond is unlikely to be cleaved during hydrolysis by trypsin, with proline's imino acid "kink-like" bond structure providing a hindrance to trypsinolysis. Trypsin's optimal performance conditions are at pH 7.8 and 37 °C (Olsen et al., 2004) although it retains varied levels of activity at other pH and temperature values as well.

For the hydrolysis of β -Lg, in addition to substrate concentration and enzyme to substrate ratio (E/S), external parameters, such as the hydrolysis pH and temperature, have to be considered. 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 pH range 7–9 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 (Pelegrine & Gasparetto, 2005). This is because the side groups of proteins are deprotonated at a high pH and protonated at a low pH to a large extent. Additionally, β -Lg undergoes autolysis under high alkaline conditions (Christen-

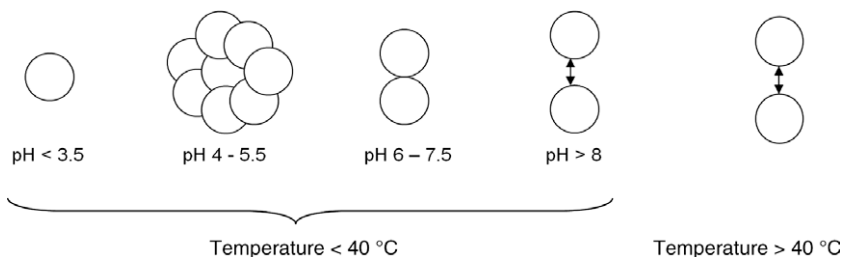


Fig. 2. Conformation of β -lactoglobulin in different environments.

sen, 1949) which process would make enzymatic cleavage less controllable.

Higher temperatures affect the solubility of proteins in two ways. One is the increased collision energy of the molecules. The other is reaction of side chain groups, which can form cross-linkages within and between peptide chains. These cross-linking reactions may reduce the protein solubility (Walstra et al., 2006). On the other hand, viscosity decreases with increasing temperature, which improves the interactions between the substrate and the enzyme. It is a commonly known phenomenon that increasing the temperature accelerates endothermic reactions. This is also true for enzymatic hydrolysis of proteins. This positive influence by the temperature is, however, reversed at much higher temperatures at which the enzyme is heat-denatured.

Although it is reported, for trypsin, that the preferred peptide bonds for its cleavage are those formed by amino acids with -amino groups (lysine and arginine), little is reported on whether this specificity is reproducible throughout all hydrolysis temperature conditions under which the enzyme stability is not adversely affected. Our work aims to reveal the influence of hydrolysis temperature beyond the effect on enzyme activity by including a thorough analysis of the hydrolysates using advanced mass spectra, as well as such influences on enzyme kinetics. Knowledge of the peptide bond preference under different hydrolysis conditions, as well as substrate pre-treatment (such as heat-denaturing or cross-linking prior to hydrolysis), is considered an important tool in the 'design' of peptides during enzyme hydrolysis.

The aim of this work, therefore, was to find out how the hydrolysis of β -Lg with trypsin may be influenced, and therefore optimised, by variations of the working temperature.

2. Materials and methods

2.1. Hydrolysis materials and equipment

Bovine β -lactoglobulin (β -Lg, N content of 96%) was prepared from whey proteins according to a procedure similar to that depicted in Fig. 3 and explained by Bramaud, Aimar, and Daufin (1997) and Gésan-Guiziou, Daufin, Timmer, Allersma, and Van Der Horst (1999). The enzyme was bovine trypsin (EC 3.4.21.4) from Sigma–Aldrich, USA with a declared activity of 5800 units/mg. Hydrolysis apparatus included an autotitrator used for the pH-stat hydrolysis (pH-Stat, TitroLine alpha plus, Schott AG, Mainz, Germany). The hydrolysis was managed using TitriSoft 2.5 (Schott AG, Mainz, Germany), run on a personal computer interfaced with the pH-stat equipment. The rest of the chemicals and equipment used are mentioned under methods.

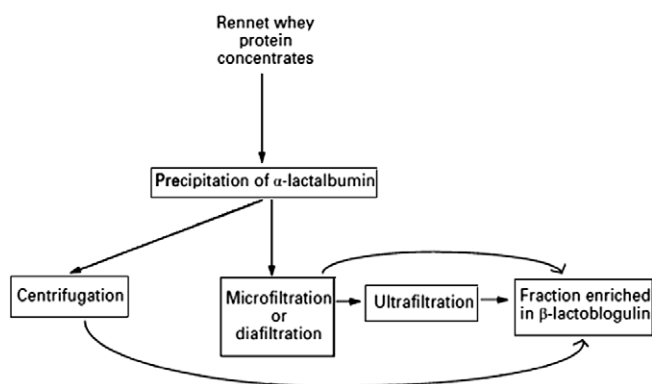


Fig. 3. Steps for the manufacture of a fraction enriched in β -lactoglobulin (Gésan-Guiziou et al., 1999).

2.2. Mass analysis materials and equipment

High purity methanol (MeOH) and ammonium acetate p.a. were purchased from Merck (Darmstadt, Germany) and high purity water from J.T. Baker (Deventer, The Netherlands). Ammonium formate and the internal standards, purine and HP-0921, were obtained from Agilent (Santa Clara, USA).

An Agilent rapid resolution high performance liquid chromatography (HPLC) system (series 1200, Waldbronn, Germany) was used for this study, in combination with a time-of-flight mass spectrometer (G1969A; 6210 TOF LC/MS, Santa Clara, USA). The apparatus consisted of a binary capillary pump (G1312B), an isocratic pump (G1310A), a degasser unit (G1379B), an auto sampler (G1367C), and a column in a thermostat set to 50 °C (G1316B). The mass-selective detector was connected by an electrospray ionisation (ESI) interface (G3251A). The HPLC, ionisation and the mass spectrometric detector were controlled and data analysed by MassHunter Workstation software (A.02.02, Agilent, Waldbronn, Germany). Tubing and connections were optimised for low dead volume performance.

2.3. Enzymatic hydrolysis of β -lactoglobulin

2.3.1. Preparation of β -lactoglobulin

The procedure of isolation of β -Lg from whey involves the removal of fats and caseins, the fractionation of the whey proteins and the purification of β -Lg. From rennet whey protein concentrate (WPC), β -Lg was isolated by the process schematically depicted in Fig. 3, following a procedure developed by Gésan-Guiziou et al. (1999) and optimised by Tolkach and Kulozik (2007) (see Fig. 3).

With the modified method, a purity of 96% β -Lg was obtained in the solution, the rest being caseinmacropeptide (CMP), a fragment from κ -casein. The liquid isolate was then dried by either freeze- or spray-drying to obtain the protein powder. The composition was roughly 50:50 between β -Lg A and β -Lg B.

2.3.2. Hydrolysis of β -lactoglobulin

Hydrolysis was performed on the lyophilised β -Lg dissolved in double-distilled water at the working pH and temperature according to the experimental design. Protein solutions were made by dissolving 6 g of β -Lg powder in 150 ml of double-distilled water to produce a substrate concentration of 4%. By addition of 3 M NaOH, the pH was adjusted to the hydrolysis pH 7.5. Even though the pH optimum for trypsin is 7.8 and not 7.5, that value was chosen because less NaOH would be required to reach the same DH which means more purity in the hydrolysate with less ash content. This pH also allowed for the elimination of the likelihood of alkaline hydrolysis of the alkaline pH-sensitive β -Lg (Christensen, 1949).

The substrate solution was equilibrated to the required temperature (30, 35, 40, 45, and 50 °C) in a thermostatically controlled, well stirred Schott Duran 300 ml jacketed-beaker glass batch reactor (HLL Landgraf Laborsysteme, Langenhagen, Germany) and hydrolysis performed using a scheme similar to that shown in Fig. 4. To maintain the pH of the solution at 7.5, the TitroLine alpha plus autotitrator was set to measure the change of pH every 10 s. The pH was kept constant with addition of NaOH (0.5 N) according to the amounts computed for each DH (Eq. (2)). The data were acquired by a personal computer connected to the autotitrator, using the TitriSoft 2.5 software. Instantaneous DH, at any given time during the hydrolysis process, was calculated using Eq. (2) according to the pH-stat method (Adler-Nissen, 1986).

When the target DH of 1%, 5% or ~9% was achieved, the hydrolysis was stopped by adding 2 M HCl to pH 3.0–3.5 (monitored using a Mettler Toledo pH-meter, Schwerzenbach, Switzerland) in order to instantaneously arrest the enzyme activity. Fifty

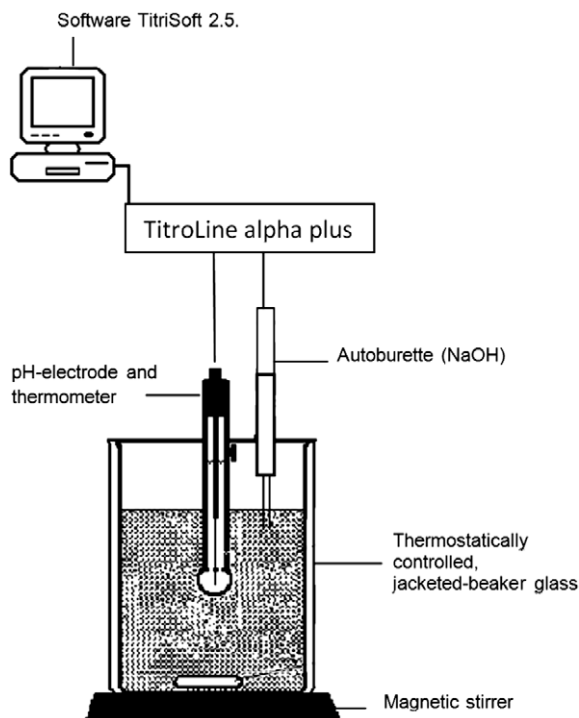


Fig. 4. Experimental set-up used in this work showing a titrator (TitroLine alpha Plus).

millilitre of the reaction volume were withdrawn and additionally heated at 95 °C for 10 min to make sure that the enzyme was completely inactivated. After cooling, the samples were frozen at –18 °C prior to further analysis. All experiments were carried out in duplicate.

2.4. Mass spectra of the hydrolysates

The liquid chromatographic separation was performed with a ProntoSil 120-3-C₁₈ column (100 × 2 mm i.d., 3 µm, Bischoff Chromatography, Leonberg, Germany). The sample injection volume was 20 µl and the mobile phase was MeOH/H₂O at a flow-rate of 200 µl/min.

The separation was performed with solution A (10 vol.% MeOH and 90 vol.% of 10 mM ammonium acetate solution, pH 7.4) and solution B (90 vol.% of MeOH and 10 vol.% of 10 mM ammonium acetate solution, pH 7.4). Prior to starting a chromatographic run, the composition of the mobile phase was kept at MeOH/H₂O 10:90 v/v, i.e. 100% solution A. Then the solution B content was increased to 30% within 1 min and finally to 100% within 8 min and held for 12 min, (with a flow-rate of 250 µl/min in the last 4 min). Then the composition was reset to 100% solution A within 15 min.

After leaving the separation column, the mobile phase was continuously mixed with 100 µl/min post-column mixing-flow. The composition of the post-column flow was 90:10 v:v MeOH/H₂O containing ammonium formate, formic acid and the mass-correcting standards purine (121.05087 Da) and hexakis(1H, 1H, 3H-tetrafluoropropoxy)phosphazine abbreviated as HP-0921 (922.00980 Da), an Agilent proprietary polyfluorinated compound, in acetonitrile:water (95:5).

The setting parameters for the ESI interface were as follows: 350 °C drying gas temperature, 6 l/min ESI drying gas flow and 45 psig ESI nebulizer gas pressure. The mass spectrometric detection was performed in positive detection mode. For the positive detection mode, a capillary voltage of 3000 V was used while the 'fragmenter' voltage of 250 V was applied. The mass-range was set to 50–3200 m/z in both detection modes, with 1.03 cycles per scan.

The peptide masses were fed into the FindPept database (<http://ca.expasy.org/tools/findpept.html>) for peptide search. FindPept identifies experimentally determined peptide masses from both specific and non-specific cleavages of a known protein. It compares measured peptide masses to all possible theoretical peptide masses that could be created from the initial protein when hydrolysed by a known enzyme such as trypsin. This allows the user to view all possible cleavage points on the original protein that would produce the experimental masses observed. For the present study, the m/z ratios for all detected ions were assumed to represent singly charged ions, and 5 ppm tolerance was allowed in the database searches. In addition, Rockefeller's PROWL (<http://prowl.rockefeller.edu/prowl/peptidemap.html>) was also used. Apart from detecting specific peptides, it is useful for detecting disulphide linkages in peptides.

2.5. Statistical analysis

The experimental design and analysis of data were done using Design-Expert® Version 6.0.11 (State-Ease, Inc., Minneapolis, MN, USA). The hydrolysis experiments were performed in duplicate runs.

3. Results and discussion

3.1. Time dependency of the DH at various temperatures

Trypsin is composed of 13 beta-strands, six of which form a beta-barrel structure. There are four regions of alpha-helix, and six disulfide bridges. The Ca²⁺-binding loop extends from Glu₇₀ to Glu₈₀. The enzymatic activity of trypsin is reported to be highly specific toward the positive side-chains of residues lysine and arginine, cleaving a peptide at the carboxyl side of these residues, during a hydrolytic reaction. The catalytic triad of trypsin forms the active site of the enzyme. Three amino acid residues, His₅₇, Asp₁₈₉, and Ser₁₉₅, are vital to the proteolytic function of the molecule. In the absence of a substrate protein, His₅₇ is unprotonated.

Bovine beta lactoglobulin (β-Lg) was fractionated from whey to obtain a product with an approximate composition of 50:50 of genetic variants A and B. Hydrolysis using trypsin was performed at temperatures from 30 to 50 °C. Trypsin optimally works at pH 7.8 and 37 °C and it was intended to investigate whether operating under stressful, i.e. non-ideal, temperatures, below or above 37 °C, could alter the enzyme hydrolytic patterns of β-Lg.

The results showed a clear dependency of the process of hydrolysis on the temperature. In Fig. 5a, it is possible to deduce that hydrolysis within the first minute showed no significant differences. A DH equivalent to 1% was attainable at all but 30 °C within the first 3 min

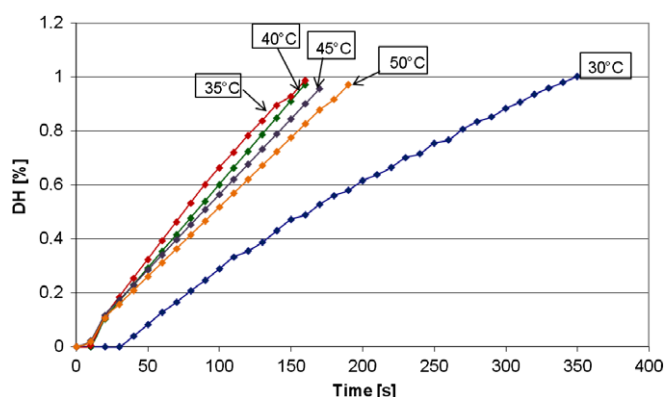


Fig. 5a. Evolution of degree of hydrolysis throughout incubation time of β-Lg with trypsin at an enzyme-to-substrate ratio of 0.05% at various temperatures (T).

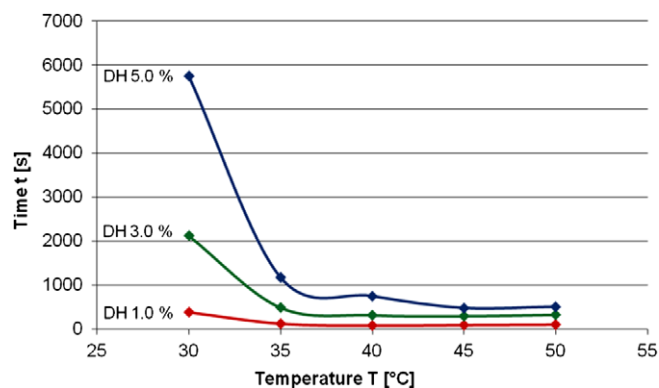


Fig. 5b. Time required for reaching a DH of 1.0%, 3.0% and 5.0%, depending on the temperature.

of hydrolysis. Hydrolysis, using trypsin at temperatures higher than the optimum 37 °C, did not slow the reaction down significantly at the initial stages of the hydrolysis. With prolonged hydrolysis times and higher DH, however, the enzyme showed a loss of activity and only marginal increases of the DH were observed. Temperatures higher than 37 °C, combined with longer hydrolysis times, led to heat-denaturation of the heat sensitive enzyme with the result that the trypsin activity declined to about 50% of the initial activity at 50 °C after 1 h of hydrolysis (results not shown).

The relationship between the hydrolysis temperature and the time required to hydrolyse β -Lg to DH values of 1%, 3% and 5% is shown in Fig. 5b. Between DH values of 1% and 5% the time difference for 30 °C is slightly less than 2 h, which decreases 11-fold at 45 and 50 °C to only <10 min. This means that the enzyme has diminished activity at temperatures below its optimum (37 °C). At temperatures higher than the optimum, the initial activity is higher (Fig. 5a) which would present challenges to enzyme control at limited hydrolysis, a key aspect of our working hypothesis. Apart from increased substrate solubility at higher temperatures, the relatively faster hydrolysis at temperatures above 35 °C could be due to the dissociation of the dimeric β -Lg into monomers (Hambling et al., 1992), leading to better accessibility of the substrate (Fig. 2) as well as near-optimal activity of the trypsin. Mota et al. (2006), reported similar trends when they hydrolysed whey protein concentrate using trypsin at 37 and 50 °C at pH 8 and pH 9. In their work, hydrolysis at 37 °C and pH 8 took longer than at pH 8 and 50 °C. In our work, with purified β -Lg as the substrate, we noted that the enzyme worked faster at 50 °C initially but the high activity was traded off with high denaturation as opposed to a relatively stable enzyme at 30–35 °C (results not shown).

Although the theoretical maximum degree of hydrolysis for trypsinolysis of β -Lg is ~10.6%, this DH was not reached throughout all experiments in this study. Additionally, after a change of the pH from 7.5 to 8.0, no significant effect on the enzyme activity was recorded. Even after hydrolysis for 26 h for one experiment, the maximum DH attained was 9.0% (85% trypsinolysis), still lower than the maximum possible DH (~10.6%). Earlier investigators obtained comparable DHs, for example those by Asselin, Amiot, Gauthier, Mourad, and Hebert (1988) and Amiot et al. (2004) who could only reach a maximum DH of 9.5%. We also found out, in our trial experiments with whey protein isolate at enzyme-to-substrate ratio of >1% (results not shown), that reaching a DH > 10.0% was increasingly difficult. Notably, work we report here constitutes some of the few instances where pure β -Lg has been hydrolysed as opposed to a mixture of proteins as is the case with either whey protein concentrate and/or whey protein isolate. Trypsin seems to have a limited ability to hydrolyse β -Lg, due to the high enzyme specificity, inhibition of trypsin by peptides (Adler-Nissen, 1986) and peptide–peptide interactions. In this regard, a DH of 5%, may

be considered as constituting about 50% trypsinolysis of β -Lg. In addition, trypsinolysis of -lactalbumin (-La), the second major protein in whey, is limited before certain DH values are reached during trypsinolysis of whey proteins. In fact this property was recently exploited by Konrad and Kleinschmidt (2008), who were able to selectively hydrolyse β -Lg, followed by membrane separation resulting in pure and native -La.

3.2. The influence of temperature on the hydrolysis products

3.2.1. General

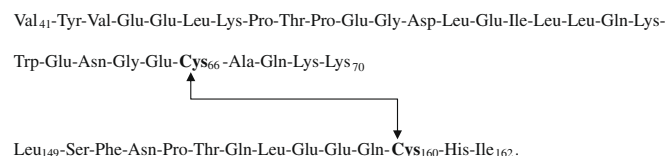
A complete analysis of the hydrolysates produced at different temperatures, using Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectra with α -cyano-4-hydroxycinnamic acid (ACHCA) as the matrix, showed that, between 30 and 40 °C, there was still some residual β -Lg after trypsinolysis to a DH of 1% (results not shown). However, at 45 and 50 °C, no residual substrate was detectable in the MALDI-TOF/MS. The reason could be the 'denaturation' of the protein at temperatures above 45 °C, due to separation of the β -Lg dimers (ideally a form of denaturation) (Hambling et al., 1992) consequently making the substrate more easily hydrolysable. Prior investigations of Ferreira et al. (2007) showed similar results, where about 42% of the initial β -Lg was intact after 180 min of incubation with trypsin at 37 °C and pH 8. This implies some substrate inhibition/resistance to trypsinolysis at this temperature.

Electrospray ionisation time-of-flight (ESI-TOF) mass spectrometry of hydrolysates produced in these experiments revealed a remarkable influence of the temperature of hydrolysis. The detection range was 50–3200 m/z , implying that detection of the intact substrate was not possible. In addition, ESI-TOF/MS, being a form of atmospheric pressure ionisation (API), is limited by the molecular weight and the hydrophobicity, and hence ionisability, of the analyte (Berkemeyer & Letzel, 2008), and it was considered meaningful to limit the range in order to optimise the information regarding low m/z species.

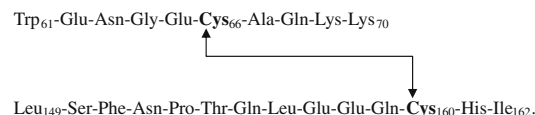
3.2.2. Influence of hydrolysis temperature at 30–35 °C on the hydrolysis products

When β -Lg was hydrolysed to a DH of 1% at 30 °C, the enzyme attacked the substrate in a manner consistent with the theoretical attack sites (Fig. 6a), namely peptide bonds formed by lysine-X and arginine-X where X is any other amino acid except proline. There were bonds whose cleavages were not detected, such as Lys-Pro, Lys-Lys, Lys-Trp, Lys-Ile and Lys-Phe, implying that they survived attack by trypsin at DH 1% when hydrolysis was performed at 30–35 °C.

The analysis of MS spectra using PROWL revealed that disulphide linkages were conserved at DH 5%, 35 °C. One of the two disulphide-linked peptides was f(41–70)-S-S-linked-to-f(149–162) with a mass of 5141.548 Da:



The second detected disulphide linked peptide was (f(61–70)-S-S-linked-to-f(149–162)). The peptide had a mass of 2847.292:



It is likely that, at other temperatures and DH 1%, the peptides existed in precursors that were not ionisable in the ESI and were thus not detected. Currently, our group is utilising MALDI-TOF/MS to analyse trypsinolysis products with the hope that those peptides which were not detected in the ESI-TOF/MS will be accounted for.

In addition, although it is possible to visualise the release of some peptides cleaved from those that were detected, those peptides were missing. Among them was an expected peptide with molecular weight of >3400 Da (f71–90). This peptide, like others, was not registered in the ESI-TOF mass spectra.

What is remarkable about the results in this study is that the hydrolysates produced at both 30 and 35 °C were strikingly similar, at least up to DH 1%, with the only difference being that the enzyme activity was markedly higher at 35 °C than at 30 °C

(Fig. 5a). It is reasonable to perform hydrolysis at 35 °C, therefore, without having any adverse effects on the peptide composition at DH 1%. No non-specific hydrolysis was detected at these temperatures at DH 1%, meaning trypsin attacked β -Lg in conformity with the hypothetical preference for peptide bonds formed by amino acids at the carboxy-terminals of lysine and arginine.

3.2.3. Influence of hydrolysis temperature at 40 °C on the hydrolysis products

Hydrolysis at 40 °C produced hydrolysates at DH 1% that looked similar to those produced at 30–35 °C except that there were additional peptides released by trypsinolysis of the peptides produced earlier (Fig. 6b). At 40 °C, trypsinolysis of β -Lg released new pep-

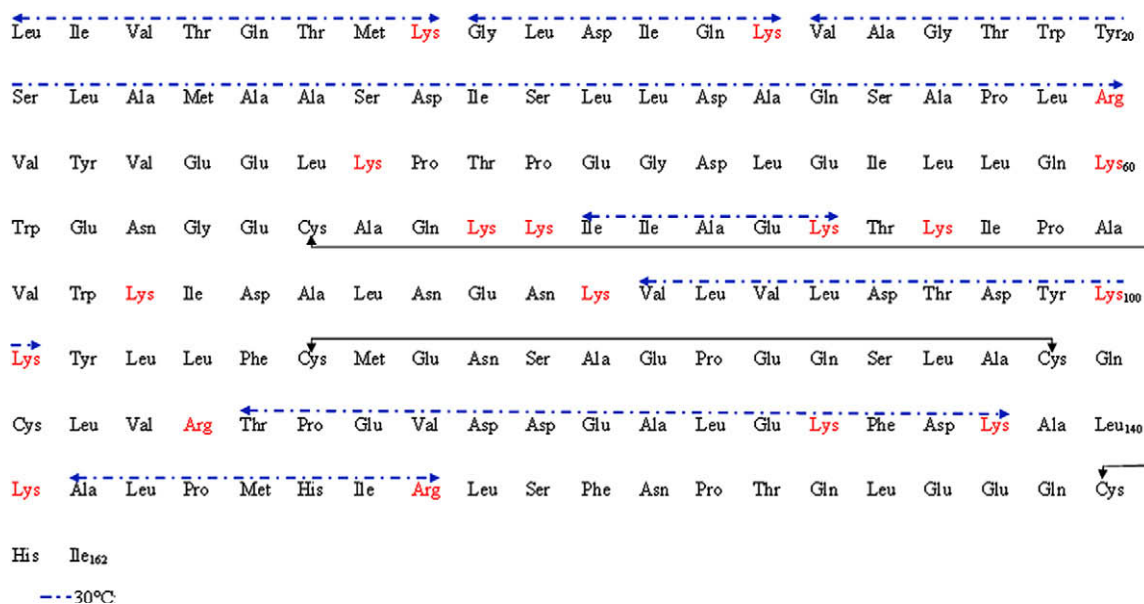


Fig. 6a. Hydrolysis pattern of β -lactoglobulin by trypsin at 30 °C, DH 1%, (Cys–Cys bridges shown by the full lines).

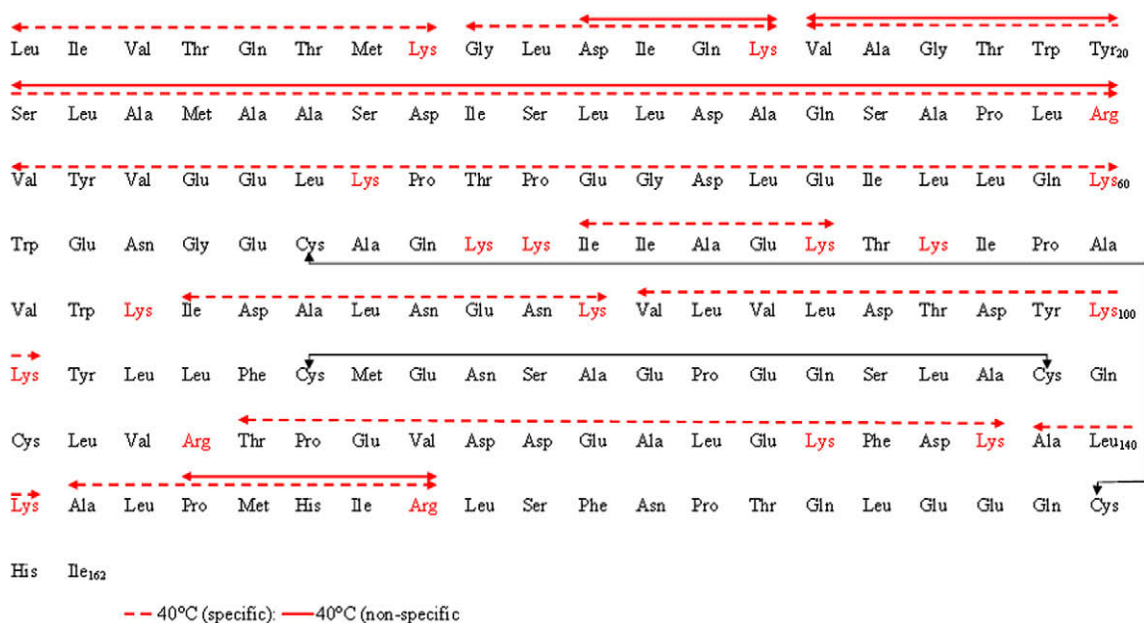


Fig. 6b. Hydrolysis pattern of β -lactoglobulin by trypsin at 40 °C, DH 1%, showing hydrolysis patterns for both specific and non-specific trypsinolysis.

tides not detected at 30–35 °C, such as f41–60, f84–90 and f139–141.

Of note is the release of peptides due to what is likely to be some non-specific hydrolysis of β -Lg by trypsin. A look at the peptide bonds cleaved to release the peptides (f11–14, f15–20, f21–40 and f144–148) reveals a tendency for trypsin to change into a chymotrypsin (3.4.21.1)-like behaviour. Trypsin cleaved Leu-X (where X was either aspartic acid or proline) and tyrosine–serine. Chymotrypsin, a serine proteinase of the digestive system like trypsin, cleaves peptides at the carboxyl side of tyrosine, tryptophan, and phenylalanine because these three amino acids contain aromatic rings, which fit into a 'hydrophobic pocket' in the enzyme. Over time, chymotrypsin also hydrolyses

other amide bonds, particularly those with leucine-donated carboxyls.

3.2.4. Influence of hydrolysis temperature at 45 °C on the hydrolysis products

Hydrolysis at 45 °C revealed a near-replication of the patterns observed at 40 °C with few exceptions for the specific hydrolysis (Fig. 6c). Principally, there appeared some new peptides, f149–162 with non-detection of the f41–60 and f71–75 peptides. Notably, at 45 °C, peptide bonds which were hydrolysable at 40 °C (Arg–Val, Arg–Thr and Lys–Ile) remained intact at 45 °C when β -Lg was hydrolysed to DH 1%.

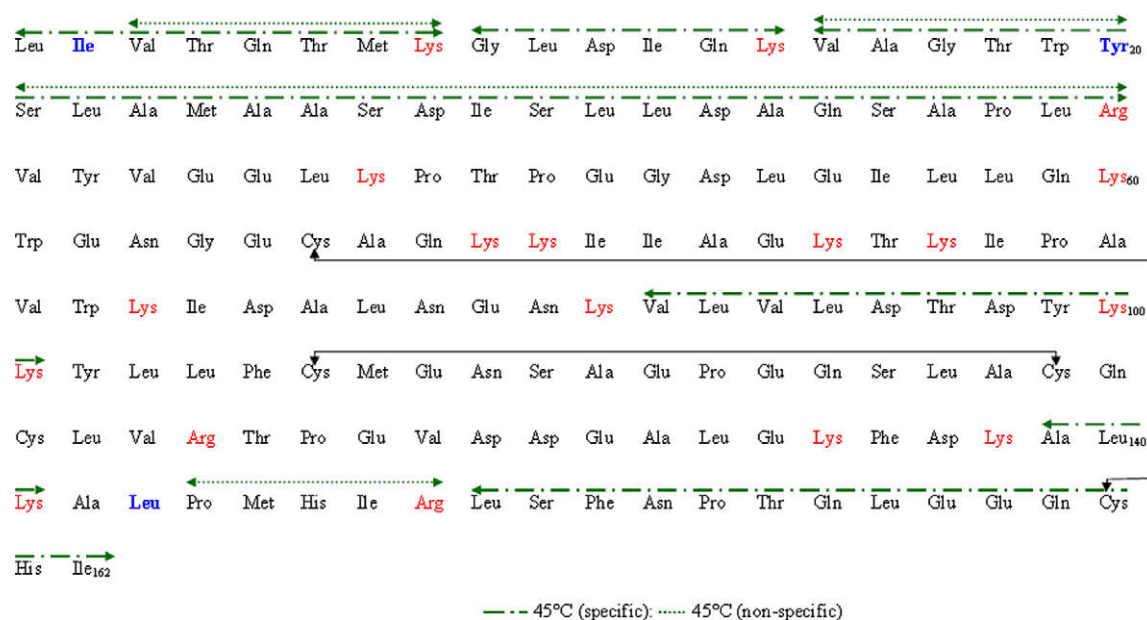


Fig. 6c. Hydrolysis pattern of β -lactoglobulin by trypsin at 45 °C, DH 1%, showing hydrolysis patterns for both specific and non-specific trypsinolysis. Lys and Arg are highlighted to show the declared preferential hydrolysis points.

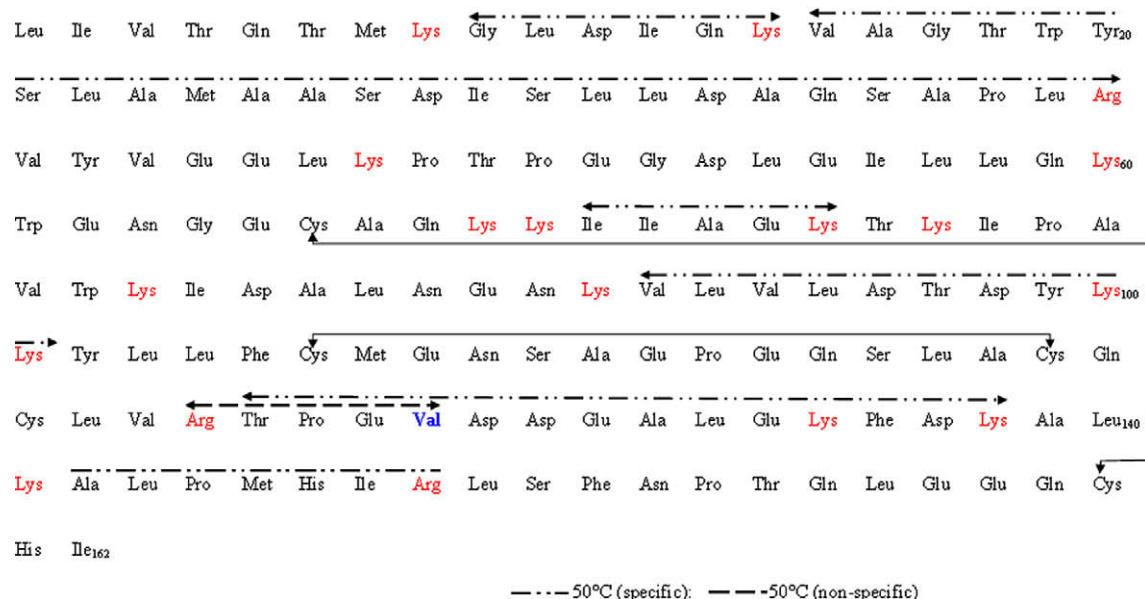


Fig. 6d. Hydrolysis pattern of β -lactoglobulin by trypsin at 50 °C, DH 1%, showing hydrolysis patterns for both specific and non-specific trypsinolysis. Lys and Arg are highlighted to show the declared preferential hydrolysis points.

The non-specific hydrolysis also revealed patterns similar to those observed at 40 °C with an additional cleavage of an Ile–Val bond (f3–8). The Leu–Asp bond was not cleaved at this temperature although the behaviour was again consistent with chymotrypsin hydrolytic patterns.

3.2.5. Influence of hydrolysis temperature at 50 °C on the hydrolysis products

Hydrolysis at 50 °C, the highest possible temperature under which trypsin could remain stable and active relatively longer, re-

vealed a diminished production of detectable peptides (Fig. 6d). For specific hydrolysis, all the peptides detected at 30–35 °C at DH 1% were also detected at 50 °C, except f1–8. Notably, the peptides were fewer than those produced at 40–45 °C at the same DH. Non-specific trypsinolysis produced only one detectable peptide, f144–148, with cleavage of Val–Arg and the typical chymotrypsin behaviour noted at 40 and 45 °C was not detected. However, at 50 °C, the chymotrypsin-like hydrolysis pattern noted at 40–45 °C was reduced probably due to a pronounced denaturation beyond mere steric re-organisation of the catalytic pocket.

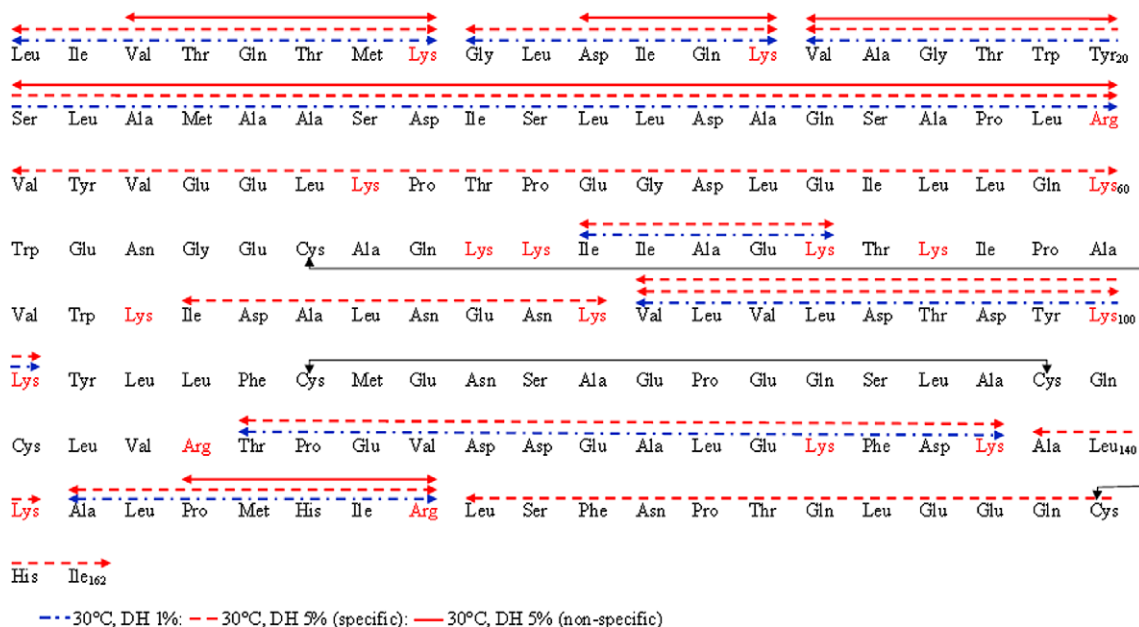


Fig. 7a. Hydrolysis pattern of β -lactoglobulin by trypsin at 30 °C, DH 1% and DH 5%, showing hydrolysis patterns for both specific and non-specific trypsinolysis at DH 5%. Lys, Arg and the non-specific cleavage points are highlighted.

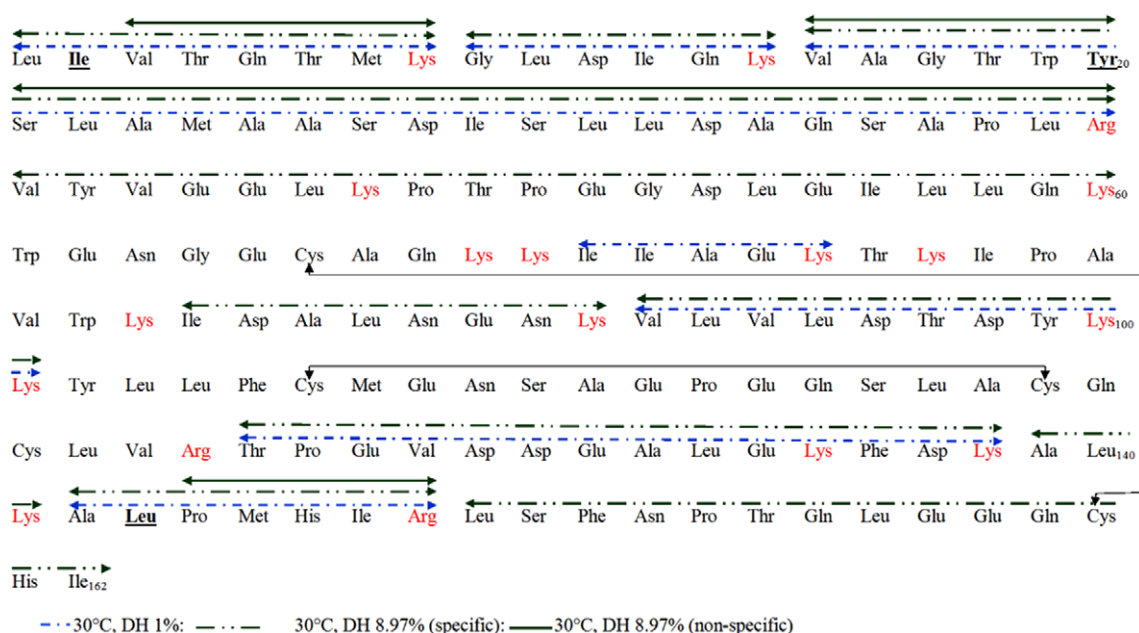


Fig. 7b. Hydrolysis pattern of β -lactoglobulin by trypsin at 30 °C, DH 1% and DH 10%, showing hydrolysis patterns for both specific and non-specific trypsinolysis at DH 5%. Lys, Arg and the non-specific cleavage points (Ile-X, Tyr-X and Leu) are highlighted.

3.3. Influence of the degree of hydrolysis at 30 °C on the hydrolysis products

Hydrolysis at 30 °C to DH 5% and ~9.0% led to the release of peptides additional to those produced at DH 1%. At DH 5%, trypsinolysis led to additional cleavage of Arg–Val, Arg–Leu, Lys–Ile and Lys–Ala bonds, yielding additional peptides for specific cleavage (Fig. 7a). In the process, the previously produced peptides were conserved under specific cleavage with the resistance of Lys–Pro, Lys–Phe and one Lys–Ile noted (Fig. 7a).

In addition to the specific cleavage, trypsinolysis followed a typical non-specific cleavage with the attack of typical chymotrypsin-like susceptible peptide bonds. The enzyme attacked Ile–Val, Leu–Asp, Tyr–Ser and Leu–Pro and in the process released peptides not associated with trypsinolysis.

Hydrolysis to a higher DH (~9.0%) (Fig. 7b), revealed a similar trend as compared to the hydrolysis to DH 5%. Specific hydrolysis yielded peptides brought about by cleavage of peptide bonds similar to those hydrolysed at DH 1% with additional cleavage of Lys–Ala, Arg–Val, Lys–Ile and Arg–Leu. Notably, resistance, even at this DH, was noticed at putatively susceptible bonds Lys–Ile (–Pro), Lys–Pro, Lys–Lys and Lys–Phe. In addition to the specific cleavage, DH ~9.0% also led to hydrolysis of bonds not classically associated with trypsinolysis, such as Ile–Val, Tyr–Ser and Leu–Pro. Notably, apart from the declared resistance of any peptide bond formed by lysine and/or arginine with proline, it was also detected that a lysine–isoleucine bond was resistant to trypsinolysis. Furthermore, the isoleucine was bonded directly to a proline, which factor may be responsible for this resistance to trypsinolysis.

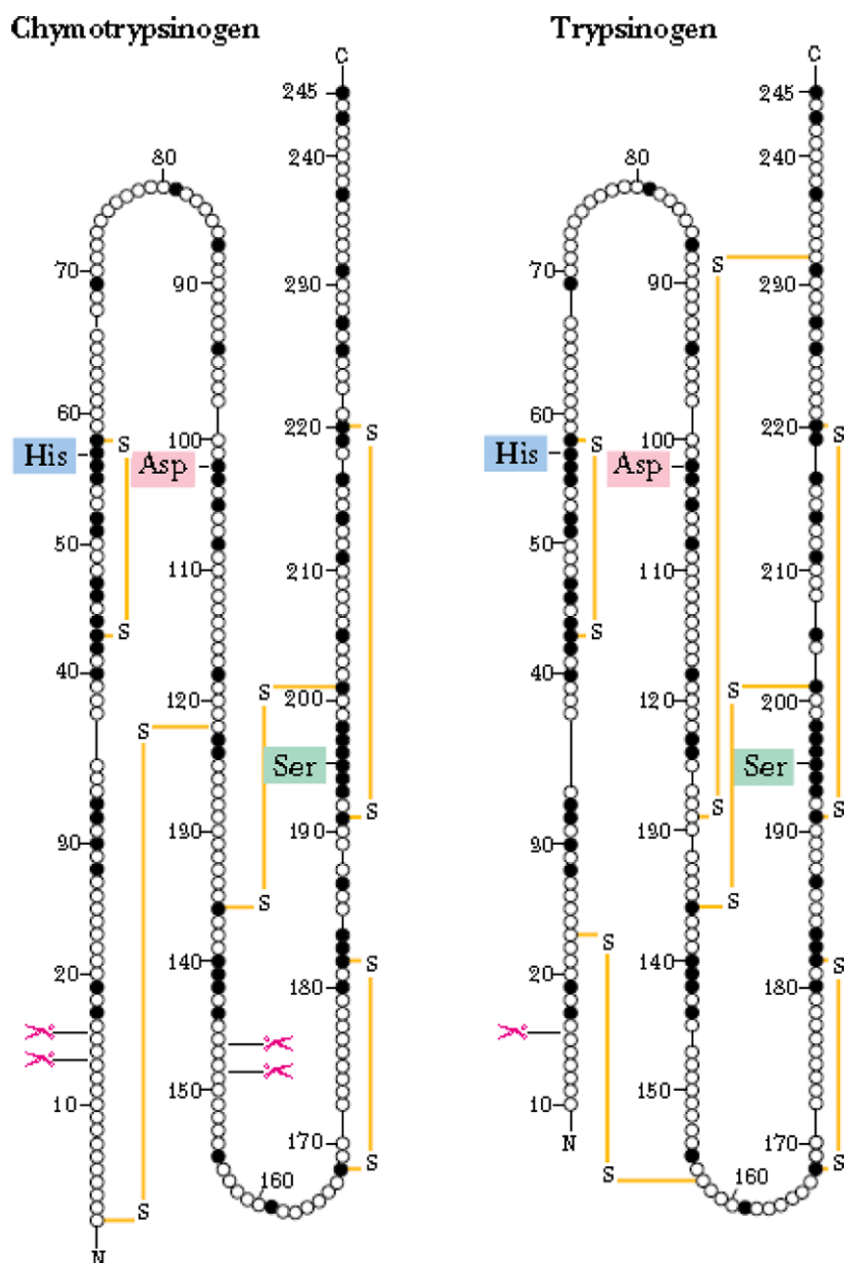


Fig. 8. Comparison of the amino acid sequences of chymotrypsinogen and trypsinogen. Each circle represents one amino acid. Numbering is based on the sequence of chymotrypsinogen. Filled circles indicate residues that are identical in the two proteins. Disulfide bonds are indicated in yellow (with S–S). The positions of the three catalytically important active-site residues (His⁵⁷, Asp¹⁰², and Ser¹⁹⁵) are indicated. Adopted from Garrett and Grisham (2007). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Trypsin retains a substantial homology to chymotrypsin (Fig. 8) with the catalytic pocket triad (His₅₇, Asp₁₀₂, Ser₁₉₅) being conserved in the two zymogens. This implicates the sharing of a primitive gene in their evolution. Trypsinolysis, following typical chymotrypsinolysis, may be accounted for by a chymotrypsin 'memory' in trypsin. At temperatures higher than its optimum, >40 °C in this study, it is expected that the catalytic pocket undergoes a conformational change, possibly leading to it being less constrained and becoming in turn slightly shallower than it normally is. This steric stretching out may allow the entry into the catalytic pocket of bulky amino acids, which are usually restricted by the tightness of the 'gate-keeping glycines' (Gly₂₁₆ and Gly₂₂₆) (Graf et al., 1988). This probably explains the apparent shift to the typical chymotrypsinolysis-like catalysis at 40–45 °C because the shallow catalytic pocket becomes large enough with more bulky amino acids as 'gate-keepers', hence allowing large amino acids entry to the hydrolytic pocket.

3.4. Undetected peptides in the ESI-TOF/MS

Electrospray ionisation time-of-flight mass spectrometry (ESI-TOF/MS) is an atmospheric pressure ionisation (API) method which is influenced to a large extent by the peptide charges. Hydrophobic peptides, for failure to ionise, would not be detected using ESI mass spectrometry (Berkemeyer & Letzel, 2008). In the course of trypsinolysis of β -Lg, it was noted that some peptides were not detectable in ESI-TOF/MS, even though a closer examination of the cleavage sites meant that the peptides were probably produced. These peptides are presented in the summary in Table 1, with their composition of hydrophobic amino acids and the calculated pI and molecular weight given, too. Of interest is a 16-amino acid residue peptide (f76–91) with a calculated molecular weight of 1840 Da, a high pI of 8.17, but a relatively high content of hydrophobic amino acids (49.8% w/w). The high content of hydrophobic amino acids may account for the non-detection of this peptide by ESI-TOF/MS, even though our work with MALDI-TOF/MS shows it to be a product of trypsinolysis of β -Lg (results not shown). Arguably this is one of the limitations of ESI as an analytical tool in high mass peptides. It is important to note, too, that even a small 14-mer peptide (1659 Da) was undetected even though its size was within the detection range. This peptide was made up of about one-third of hydrophobic amino acids. The non-detection of certain peptides in β -Lg hydrolysates should invite a re-examination of ESI-TOF/MS as a stand-alone analytical tool where a complete catalogue of the hydrolysis products is desired. At the moment, our group is comparing the information ob-

tained from MALDI-TOF/MS in order to identify any deviations that there may be.

4. Conclusions

The activity of the trypsin revealed depressed trypsinolysis of β -Lg at 30 °C, while the activity was higher at 40 °C. The initial hydrolytic activities at all the screened temperatures were similar, with variations in the activity showing over longer hydrolysis times and higher DH values. The bonds formed by lysine and proline, isoleucine (followed by proline) and phenylalanine remained resistant to trypsinolysis at all temperatures of hydrolysis.

Analysis of the hydrolysates by electrospray ionisation time-of-flight mass spectrometry (ESI-TOF/MS) revealed a similarity at DH 1% between the hydrolysates produced at 30 and 35 °C, meaning that the enzyme retained its unique trypsin characteristic at those temperatures, too. Disulphide-linked peptides were detectable at 35 °C for hydrolysates produced at DH 5%. At 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 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.

Our results provide both a warning and an opportunity: in order to operate under trypsin-like conditions, the sensitivity of the enzyme to the temperature needs to be considered. This deviation by trypsin may, however, provide an opportunity to steer the enzyme to behave in a unique way and hence enable us to design peptides with targeted amino acid composition at the specific termini.

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Table 1
Peptides undetected but probably released by trypsinolysis of β -lactoglobulin.

Fraction	Peptide ^a	MW ^b (Da)	Calculated pI	H ₀ AA ^c (%)
f41–70 (30-mer) ^d	VYVEELKPTPEGDLEILLQK	3487.9	4.6	39.9
f76–91 (16-mer)	TKIPAVVWKIDALNENK	1840.1	8.2	49.8
f102–124 (23-mer)	YLLFCMENSAPPEQSLACQCLVR	2676.1	4.3	43.3
f149–162 (14-mer)	LSFNPTQLEEQCHI	1658.8	4.5	35.6

^a Single letter amino acid symbols used.

^b Molecular weight.

^c Content of hydrophobic amino acids (alanine (A), isoleucine (I), leucine (L), phenylalanine (F), Proline (P), tryptophan (W), tyrosine (Y) and valine (V)) where present.

^d Number of amino acids given as '-mer'.

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