



Influence of buffer type and concentration on the peptide composition of trypsin hydrolysates of β -lactoglobulin

Seronei Chelulei Cheison^{a,b,*}, Elena Leeb^a, Thomas Letzel^c, Ulrich Kulozik^d

^a Zentralinstitut für Ernährungs und Lebensmittelforschung (ZIEL) – Junior Research Group, Bioactive Peptides and Protein Technology, Technische Universität München, Weihenstephaner Berg 1, D-85354 Freising, Germany

^b School of Public Health and Community Development, Maseno University, Private Bag, Kisumu, Kenya

^c Analytical Research Group, Chair of Biopolymer Chemistry, Technische Universität München, Freising-Weihenstephan, Germany

^d Chair for Food Process Engineering and Dairy Technology Department, ZIEL Section Technology, Technische Universität München, Weihenstephaner Berg 1, D-85354 Freising, Germany

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ABSTRACT

Proteins acquire conformation in aqueous media due to the influence of the buffer salt composition and concentration. Such influence may have impact on the enzyme–substrate interaction and somehow steer the enzyme attack properties, leading to release of dissimilar products. Our group has sought to investigate the influence of the hydrolysis environment on the trypsinolysis of a model protein, β -lactoglobulin (β -Lg). This work was aimed at investigating the effect of different buffers and their concentrations on the trypsinolysis patterns of β -Lg. The traditional NaOH-buffered water, in comparison to Tris–HCl and potassium-phosphate buffer at 62.5 mM–1.0 M were used at pH 8.5 for the pH drop and pH 7.8 for the hydrolysis. Bovine trypsin (EC 3.4.21.4) was used at an enzyme-to-substrate ratio of 1%. The samples were analysed for mass composition, using LC-ESI-TOF/MS and MALDI-TOF/MS for monitoring time-dependence of peptide evolution. In all buffer types and concentrations, peptides $f(1-8)$, $f(15-40)$, $f(125-138)$ and $f(142-148)$ were detected, implicating ease of hydrolysis of the terminal regions of β -Lg. A peptide from $f(9-14)$, with sequence Gly-Leu-Asp-Ile-Gln-Lys, was detected at >0.5 M Tris–HCl only, while peptide $f(71-75)$ was unique to <125 mM Tris–HCl and >250 mM potassium-phosphate buffer. Hydrolysis under buffer produced trypsin-specific peptides, numerous chymotrypsin-like non-specific peptides but no disulphide-linked peptides. Trypsinolysis shifted to the N-terminal region of lysine under some conditions. Hydrolysis under buffer holds potential for the avoidance of some peptides with undesirable characteristics while preserving a diversity of different peptides with possible bioactive properties.

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1. Introduction

Whey protein β -lactoglobulin (β -Lg) is a small, soluble protein with a globular structure and a monomer molecular weight of 18.4 kDa. Its primary structure consists of 162 amino acids, of which 15 are lysine, and three are arginine residues, potential attack sites for trypsin (Fig. 1). Access by the enzyme to the highlighted cleavage sites in Fig. 1 is influenced by the solvent media effects on the protein conformation (both the substrate and enzyme). This effect might be caused by the charge density around the proteins as well as the effect of specific buffer salts.

Bovine trypsin (EC 3.4.21.4) is a serine protease (molecular weight of 23.3 kDa) with a nucleophilic active site with an aspartic acid sitting in a specificity pocket (position 189). It cleaves predominantly at the positively charged side chains of arginine–X

and lysine–X, with X being any amino acid except proline (Olsen, Ong, & Mann, 2004). We reported earlier on the resistance of β -Lg to trypsin at peptide bonds formed by neighbouring lysines (Lys–Lys), Lys–Ile(–Pro), Lys–Pro and Lys–Phe during trypsinolysis at different temperatures (Cheison, Schmitt, Leeb, Letzel, & Kulozik, 2010).

The interactions between proteins and the solvent media are complex. 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 in a favourable medium. Relative to the strength of water–water interactions in 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

* Corresponding author. Tel.: +49 (0) 816171 5056; fax: +49 (0) 816171 4384.

E-mail address: Seronei.Cheison@wzw.tum.de (S.C. Cheison).

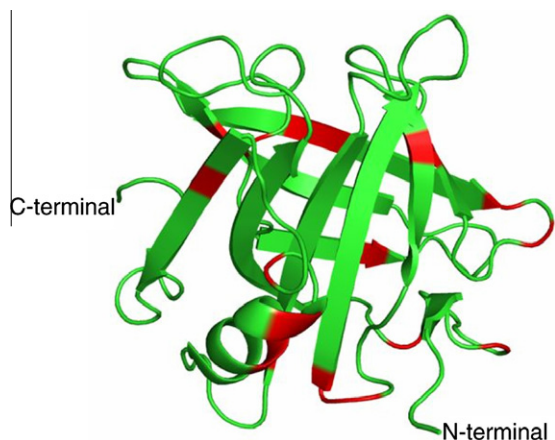


Fig. 1. Molecular structure of β -lactoglobulin (β -Lg) with preferential cleavage sites of trypsin highlighted in red. (Compiled on <http://polyview.cchmc.org> on December 4th, 2008). The trypsin-scissile bonds are Lys⁸-Gly⁹, Lys¹⁴-Val¹⁵, Arg⁴⁰-Val⁴¹, Lys⁴⁷-Pro⁴⁸, Lys⁶⁰-Trp⁶¹, Lys⁶⁹-Lys⁷⁰, Lys⁷⁰-Ile⁷¹, Lys⁷⁵-Thr⁷⁶, Lys⁷⁷-Ile⁷⁸, Lys⁸³-Ile⁸⁴, Lys⁹¹-Val⁹², Lys¹⁰⁰-Lys¹⁰¹, Lys¹⁰¹-Tyr¹⁰², Arg¹²⁴-Thr¹²⁵, Lys¹³⁵-Phe¹³⁶, Lys¹³⁸-Ala¹³⁹, Lys¹⁴¹-Ala¹⁴² and Arg¹⁴⁸-Leu¹⁴⁹. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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 & 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 (Yang, 2009). Chaotropic anions are water-structure breakers and destabilize proteins. Kosmotropic anions are polar water-structure 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, Hendle, and Schmid (1997) have shown that increasing the acetate or phosphate buffer concentration from 50 mM to 1 M caused a three- and 10-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 buffer at 65 °C. The heat-stability of the enzyme originates from its ability to refold completely into the native-like state, fully stabilizing the native protein, in which coulombic interactions are reduced at low ionic strength (Dominy, Perl, Schmid, & Brooks, 2002). These two examples underscore the role of buffer concentration and salt types in the protein stability.

Although the molecular dimer-monomer dissociation of β -Lg was demonstrated to be dependent on, and to be favoured by, the pH (>pH 7.5) and temperature (>40 °C) (Pessen, Purcell, & Farrell, 1985; Tanford, Bunville, & Nozaki, 1959), the effect of the buffer concentrations on the equilibrium between dimers and monomers is still unclear. Decreased ionic strength and temperature, for example favour oligomerisation of β -Lg at pH 4.65 (Verheul, Pedersen, Roefs, & de Kruif, 1999). Dimerisation is favoured by changes in the pH towards the pI, as well as an increase in the ionic strength (Vardhanabhuti & Foegeding, 2008), and is influ-

enced by protein concentration. We have demonstrated (in a recent study) that, using the hydrolysis medium to break up the dimers to monomers in β -Lg, shifted the hydrolysis preference from random attack of whey proteins to selective trypsinolysis of β -Lg.

Our hypothesis is that the hydrolysis environment drives the protein and enzyme interaction due to changes it induces on their spatial conformation. This is postulated to affect the access of the enzyme to the scissile bonds, which may lead to alteration of the enzyme behaviour and attack pattern, resulting in the release of different peptides. In this way, some peptides which could not be produced in a normal water-NaOH/HCl buffered system might be released while some might not, which has an impact on the peptide composition. The aim of this work was to reveal the effects of buffer type and concentration on the trypsinolysis patterns of β -Lg in order to determine all the possible peptides released under each working condition.

2. Materials and methods

2.1. Hydrolysis materials and equipment

Most of the materials used were reported in our earlier work (Cheison et al., 2010). Bovine β -Lg (protein content of 96%) was prepared from whey protein isolate (WPI) bought from Fonterra Co-operative Group Ltd., Auckland, New Zealand. Bovine trypsin (EC 3.4.21.4) was from Sigma-Aldrich, MO, USA with a declared activity of 5800 units/mg. Hydrolysis apparatus for the simulated pH-stat method included an autotitrator (pH-Stat, TitroLine alpha plus, Schott AG, Mainz, Germany). The hydrolysis was managed using TitriSoft 2.5 software (Schott AG, Mainz, Germany), run on a personal computer inter-phased with the pH-stat equipment. The rest of the chemicals and equipment used are mentioned under methods.

2.2. Enzymatic hydrolysis of β -lactoglobulin

2.2.1. Preparation of β -lactoglobulin

From rennet whey protein concentrate (WPC), β -Lg was isolated by the process developed by Gésan-Guizieu, Daufin, Timmer, Allersma, and Van Der Horst (1999) and optimised by Tolkach and Kulozik (2007).

2.2.2. Buffering strength of Tris-HCl and potassium-phosphate buffers

To investigate the buffering stability of the buffers, hydrolysis was performed on the lyophilised β -Lg dissolved in the working buffer at the working pH, buffer concentration and temperature, according to the experimental design. Protein solutions were made by dissolving 2.5 g of β -Lg powder in 25 ml of the working buffer (50 ml Eppendorf tubes with screw-caps) to make a substrate concentration of 10% (w/v). The buffers were Tris-HCl, pH 8.5 (1, 0.5, 0.25, 0.1 and 0.0625 M) and potassium-phosphate buffer, pH 8.0 (1, 0.5, 0.25, 0.125 and 0.0625 M). Hydrolysis was also performed in NaOH-buffered water at pH 7.8 and 8.5 as reference buffer.

The pH was monitored during the hydrolysis in Eppendorf tubes, using a SevenGo™ pH meter equipped with a 3 mm diameter InLab® pH electrode (Mettler Toledo AG, Schwerzenbach, Switzerland). Hydrolysis was performed at 37 ± 0.1 °C in all buffers with the tubes securely suspended in a thermostatically controlled water bath and stirring provided with a magnetic stirrer from below. Hydrolysis was commenced with the addition of bovine trypsin (EC 3.4.21.4, Sigma Aldrich, St. Louis, MO, USA) to an effective enzyme-to-substrate ratio (E/S) concentration of 1% (w/w). The pH was allowed to drop (uncontrolled) for 30 min with readings at 5 min intervals.

To monitor the peptide evolution trends in the Tris–HCl buffer, aliquots of reactor contents were withdrawn just before the addition of the enzyme, 5 min after the commencement of trypsinolysis and at 5 min intervals thereafter until the end of hydrolysis. The enzyme activity was stopped in each case by adjusting the pH to <3.5 with 2 M HCl and the samples were frozen at -18°C until further analysis.

2.2.3. The pH-stat-controlled hydrolysis

To simulate the pH-stat method, 10% (w/v), β -Lg substrate solution (100 ml) were made in the respective buffers (Tris–HCl and potassium-phosphate buffer) at 62.5 mM to 1 M concentrations were made in a 150 ml thermostatically controlled, well stirred Schott Duran jacketed-beaker glass batch reactor (HLL Landgraf Laborsysteme, Langenhagen, Germany). The substrate was also dissolved in the working buffer and the pH adjusted to 7.8. The enzymatic reaction was commenced with the addition of 1% E/S (w/w) trypsin while the pH was controlled at 7.8 by addition of 0.5 M NaOH, using an autotitrator for the pH-stat hydrolysis (pH-Stat, TitroLine alpha plus, Schott AG, Mainz, Germany). Hydrolysis was managed using TitrSoft 2.5 (Schott AG, Mainz, Germany), run on a personal computer inter-phased with the pH-stat equipment. The amount of NaOH used to maintain the pH was converted to 'apparent DH' using Eq. (1) (Adler-Nissen, 1986).

$$\text{DH} = \frac{V_B \cdot N_B \cdot 100\%}{\alpha \cdot \text{MP} \cdot h_{\text{tot}}} \quad (1)$$

where V_B = base consumption in ml, N_B = normality of the base, α = the average degree of dissociation of the α -NH groups, MP = mass of protein in g, h_{tot} = total number of peptide bonds in the protein substrate (7.2 meqv/g protein in this work) (Cheison et al., 2010).

Just before the addition of trypsin, a 0.5 ml aliquot was withdrawn from the β -Lg solution and mixed with an appropriate amount of 2 M HCl. After 1 h of hydrolysis, 0.5 ml aliquots were similarly withdrawn and the enzyme activity stopped with 100 μl of 2 M HCl. The instantaneous DH at any time during hydrolysis was calculated using Eq. (1) according to the pH-stat method (Adler-Nissen, 1986). After 1 h of reaction, the remaining reaction mixture was similarly mixed with 2 M HCl and stored frozen at -18°C for further analysis. All experiments were carried out in duplicate.

2.3. LC-ESI-TOF/MS spectra of the hydrolysates

The liquid chromatography electrospray source ionisation time-of-flight mass spectrometry (LC-ESI-TOF/MS) was performed as explained previously (Cheison et al., 2010) with a ProntoSIL 120-3-C₁₈ column (100 mm $\times$ 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 $\mu\text{l}/\text{min}$. 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, CA, 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 thermostat set at 50°C (G1316B). The mass-selective detector was connected by an ESI interface (G3251A). The HPLC, ionisation and the mass spectrometric detector were controlled and data were analysed by MassHunter Workstation software (A.02.02, Agilent, Waldbronn, Germany). Tubing and connections were optimised for low dead volume performance.

The peptide masses were fed into FindPept database (<http://ca.expasy.org/tools/findpept.html>) and PROWL's peptidemap

(<http://prowl.rockefeller.edu/prowl/peptidemap.html>) for peptide search and disulphide linkage identification.

2.4. MALDI-TOF/MS spectra of the hydrolysates

Samples collected at time intervals were also analysed for mass composition of the hydrolysates, using a matrix assisted laser desorption/ionisation time-of-flight mass spectrometer, MALDI-TOF/MS (ultrafleXtreme™ MALDI-TOF/TOF, Bruker Daltonics, Bremen, Germany). Four spots (0.5 μl) of the analytes diluted 1:100 to achieve a protein range of 20 pmol/ μl were deposited on the Prespotted AnchorChip™ PAC 384/96 disposable target plates holding 96 sample spots centred on the target and prespotted with an α -cyano-4-hydroxycinnamic acid (HCCA) matrix prepared with 24 calibrant spots. The nine calibrants (1–10 fmol each) were Bradykinin Fragment 1–7 (757.3992 Da), Angiotensin II (1046.5418 Da), Angiotensin I (1296.6848 Da), Neurotensin (1672.91700 Da), Renin Substrate (1758.9326 Da), ACTH clip 1–17 (2093.0862 Da), ACTH clip 18–39 (2465.1983 Da), ACTH clip 1–24 (2932.5879 Da) and ACTH clip 7–38 (3657.9289 Da). The covered mass range offered by the calibration standard is ~ 700 –4000 Da.

The MALDI-TOF/MS was run in positive reflectron (RP) mode, managed using flexControl™ 3.0 Software (Bruker Daltonics, Bremen, Germany). The FlexControl 3.0 method "RP-pepmix.par" for peptide analysis was chosen and modified, which was run in autoMS. The laser strength was optimised at 35% above threshold. The peaks were picked using FlexAnalysis 3.0, submitted to BioTools 3.2 build 3.2.1.31 all from Bruker Daltonics (Bremen, Germany). Only those peptides which were common to the four spots were considered safe to assign a sequence.

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. Buffering stability

The performance stability of the buffers was analyzed on the basis of the pH-drop witnessed in the first 30 min of the reaction following addition of trypsin (Cheison, Wang, & Xu, 2007). As expected, the buffering stability was largely dependent on the buffer concentration, in each case, with the ΔpH in the buffered reactions being lower than when NaOH-buffered water was used. When seeking to produce a hydrolysate with less ash content, therefore, the least possible buffer concentration needed to achieve stable buffering may be chosen. The objective in our work, however, was to determine influence of the buffering salt and its concentration on the trypsinolysis patterns of bovine β -Lg. The buffer concentration (up to 1 M) was thought to induce conformational changes to the protein and the enzyme molecules apart from possibly influencing the medium in which the protons are exchanged during catalysis. It is also likely that the increased buffering had an impact on the susceptibility of the protein to denaturing under the hydrolysis conditions. Those changes might have a marked influence on the kinetics of enzyme–substrate interaction and therefore the peptides produced, apart from the long-term stability of the substrate and the enzyme.

Fig. 2 shows a modification of the normal DH curve during hydrolysis in a potassium-phosphate buffer. The pH change was

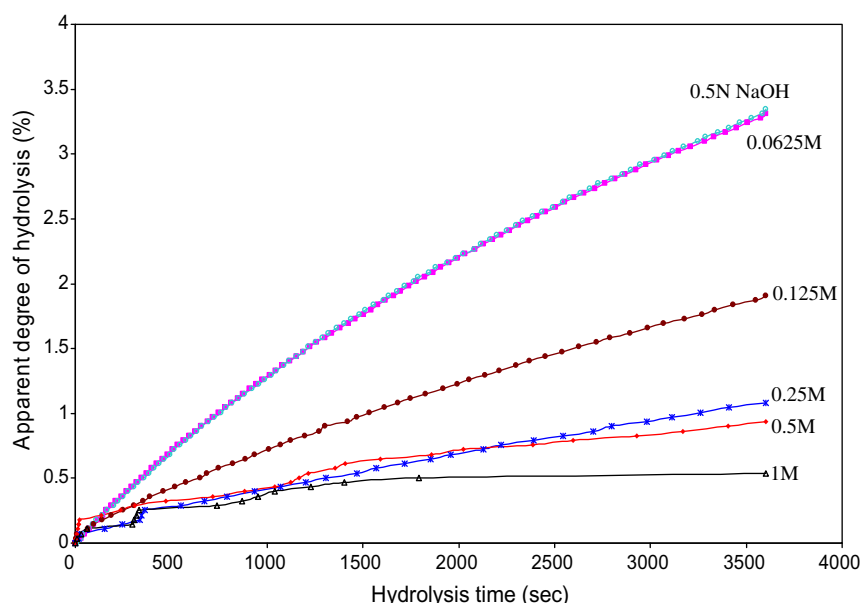


Fig. 2. Simulated pH-stat hydrolysis in potassium-phosphate buffer from 62.5 mM to 1 M compared with NaOH-buffered hydrolysis in water, showing the buffering capacity during 60 min hydrolysis of β -Lg.

corrected using 0.5 M NaOH. In this curve, the apparent DH, being a conversion of the volume of 0.5 M NaOH required to stabilise the pH during the 60 min, was calculated using Eq. (1). Ideally, a hydrolysis mixture with stable buffering required less NaOH and, therefore, a low apparent DH was attained. This is observed for 1 M potassium-phosphate buffer. With the lowest buffer concentration (62.5 mM), the amount of NaOH used to regulate the pH tallies closely with that used when NaOH-buffered water was used, implying a similar buffering strength between the 62.5 mM potassium-phosphate buffer and 0.5 M NaOH. This water (weak acid)-NaOH (weak base) system had weak buffering capacity on its own (results not shown). One of the problems detected with the use of NaOH during mass spectrometry of hydrolysates was the formation of sodium adducts (+22 Da masses) which is likely to be obviated with the use of (semi)-volatile buffers such as ammonium bicarbonate, a buffer which was also tried in our work (results not shown).

3.2. Mass spectrometry of peptides: specific peptides

The evolved peptides in the hydrolysates of bovine β -Lg under buffers were identified using LC-ESI-TOF/MS. Peptides which were released through trypsinolysis of peptide bonds, formed by other amino acids on the carboxy-terminals of Lys and Arg, were considered to be due to the specific activity of trypsin and therefore categorised as specific peptides. Table 1 presents a summary list of the specific peptides identified in Tris-HCl and potassium-phosphate buffers. When the hydrolysis was performed in potassium-phosphate buffer for example, $f(9-14)$ (made up of amino acid sequence Gly-Leu-Asp-Ile-Gln-Lys) was not detected. The peptide was detected in the hydrolysates produced in Tris-HCl at 0.5 and 1 M. This peptide was earlier reported to have angiotensin-I converting enzyme (ACE) inhibitory properties (Pihlanto-Leppälä, Rokka, & Korhonen, 1998), as well as being hypocholesterolaemic (Nagaoka et al., 2001). A potassium-phosphate buffer unique peptide, $f(70-$

Table 1
Peptides produced due to specific trypsinolysis of bovine β -Lg in Tris-HCl (Tris)/potassium phosphate (KP) (1 h).

Fraction	Observed mass (Da)	Calculated mass (Da)	Sequence	0.0625 M		0.125 M		0.25 M		0.5 M		1 M		NaOH DH 3.32%
				Tris ^a	KP ^b	Tris	KP	Tris	KP	Tris	KP	Tris	KP	
$f(1-8)$	936.536	936.540	Leu-Ile-Val-Thr-Gln-Thr-Met-Lys	+	+	+	+	+	+	+	+	+	+	+
$f(9-14)$	672.383	672.381	Gly-Leu-Asp-Ile-Gln-Lys	—	—	—	—	—	—	+	—	+	—	—
$f(15-40)$	2706.358	2706.369	Val-Ala-Gly-Thr-Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile-Ser-Leu-Leu-Asp-Ala-Gln-Ser-Ala-Pro-Leu-Arg-Val-Tyr-Val-Glu-Glu-Leu-Lys-Pro-Thr-Glu-Gly-Asp-Leu-Glu-Ile-Leu-Leu-Gln-Lys	+	+	+	+	+	+	+	+	+	+	+
$f(41-60)$	2312.247	2312.251	Lys-Ile-Ile-Ala-Glu-Lys	—	+	—	—	—	—	+	—	—	—	—
$f(70-75)$	700.449	700.448	Ile-Ile-Ala-Glu-Lys	+	—	+	—	—	+	—	+	—	+	—
$f(71-75)$	572.355	572.353	Ile-Ile-Ala-Glu-Lys	+	+	+	—	+	+	+	+	—	+	+
$f(84-91)$	915.470	915.466	Ile-Asp-Ala-Leu-Asn-Glu-Asn-Lys	+	+	+	+	+	+	+	+	+	+	+
$f(92-100)$	1064.578	1064.575	Val-Leu-Val-Leu-Asp-Thr-Asp-Tyr-Lys	+	+	+	+	+	+	+	+	+	+	+
$f(92-101)$	1192.671	1192.670	Val-Leu-Val-Leu-Asp-Thr-Asp-Tyr-Lys-Lys	+	+	+	+	+	—	+	+	+	+	+
$f(125-138)$	1634.769	1634.767	Thr-Pro-Glu-Val-Asp-Asp-Glu-Ala-Leu-Glu-Lys-Phe-Asp-Lys	+	+	+	+	+	+	+	+	+	+	+
$f(142-148)$	836.470	836.469	Ala-Leu-Pro-Met-His-Ile-Arg	+	+	+	+	+	+	+	—	+	+	+
$f(149-162)$	1657.777	1657.777	Leu-Ser-Phe-Asn-Pro-Thr-Gln-Leu-Glu-Gln-Cys-His-Ile	+	+	+	+	+	+	+	—	—	—	+

^a Tris-HCl buffer.

^b Potassium phosphate buffer.

75), with the sequence Lys-Ile-Ile-Ala-Glu-Lys, was detected. A fragment of that peptide, f(71–75), was detectable at lower concentrations of Tris–HCl (62.5 and 125 mM) and higher concentrations of potassium-phosphate buffer (0.25–1 M).

Trypsinolysis of β -Lg was found to cleave the Lys⁸–Gly⁹ in all buffers and in all buffer concentrations, to release the fragment f(1–8). This peptide was identified earlier even when trypsinolysis was performed at different temperatures (Cheison et al., 2010), implying that it was probably among the first products of β -Lg trypsinolysis. Other peptides identified in all buffers are fragments f(15–40) and fragment f(125–138), made up of Thr¹²⁵–Pro–Glu–Val–Asp–Asp–Glu–Ala–Leu–Glu–Lys–Phe–Asp–Lys¹³⁸.

Another fragment, f(142–148), with the sequence Ala¹⁴²–Leu–Pro–Met–His–Ile–Arg¹⁴⁸ was also identified, except in 0.5 M potassium-phosphate buffer. Another common product of trypsinolysis, the carboxy-terminal fragment, f(149–162), with sequence Leu¹⁴⁹–Ser–Phe–Asn–Pro–Thr–Gln–Leu–Glu–Gln–Cys–His–Ile¹⁶², was missing at higher concentrations of the buffer.

As is evident from Table 1, the specific peptides released in different buffers were present in the NaOH-buffered water with a close resemblance to the products of hydrolysis in 62.5 mM Tris–HCl buffer. Resistance was detected in Lys⁴⁷–Pro⁴⁸, Lys⁷⁷–Ile⁷⁸–Pro⁷⁹ and Lys¹³⁵–Phe¹³⁶. The Pro⁷⁹, contributed to the resistance of the Lys⁷⁷–Ile⁷⁸ trypsin-scissile bond to trypsinolysis, even though the enzyme began to hydrolyse other bonds not associated with trypsinolysis (results not shown). Cleavage of β -Lg, at Arg¹⁴⁸–Leu¹⁴⁹ within a β -strand, yielded a peptide, f(149–162), produced at <1 M in both buffers. No disulphide-linked peptides were detected in the hydrolysates under buffer and NaOH-buffered water at the DH of 3.3%. One possible explanation for this is that the disulphide-linked peptides existed in large polypeptide forms which were not easily ionised for ESI analysis, a phenomenon we have witnessed previously (Cheison et al., 2010).

3.3. Non-specific peptides

Trypsin has been noted to release peptides due to cleavage of peptide bonds other than those formed by Lys and Arg with other amino acids. This peculiar property was recognised as being due to non-specific activity and the products are therefore categorised as non-specific peptides. Analysis of the data shows that the N-terminal of β -Lg was generally more susceptible to trypsinolysis than was the C-terminal.

A shift from the trypsinolysis of the carboxy- to the N-terminal of Lys was noted with the release of sequences f(8–13), involving the cleavage of the N-terminal of Lys⁸ and Lys¹⁴, a property called semi-trypsinolysis. This peptide was released at high (>0.5 M) Tris–HCl concentration but was not detected under other buffer conditions. Other peptide bonds cleaved at the N-terminal of Lys include Leu⁴⁶–Lys⁴⁷, Glu⁷⁴–Lys⁷⁵, Trp⁸²–Lys⁸³, Asn⁹⁰–Lys⁹¹ and Tyr⁹⁹–Lys¹⁰⁰.

This property resembles attack of chymotrypsin-scissile bonds; α -chymotrypsin catalyses the hydrolysis of peptide bonds on the C-terminal side of tyrosine, phenylalanine, tryptophan and leucine. Secondary hydrolysis will also occur on the C-terminal side of methionine, isoleucine, serine, threonine, valine, histidine, glycine and alanine. Trypsinolysis of typical chymotrypsin scissile peptide bonds, such as the carboxy-termini of Leu-(f41–57) and f(144–148) with sequence Pro¹⁴⁴–Met–His–Ile–Arg¹⁴⁸, were identified in the buffers. In addition, typical chymotrypsin-like hydrolysis occurred at the carboxy-terminals of tyrosine (Tyr²⁰–Ser²¹ and Tyr⁹⁹–Lys¹⁰⁰), tryptophan (Trp¹⁹–Tyr²⁰ and Trp⁸²–Lys⁸³) and leucine (Leu¹–Ile², Leu²²–Ala²³, Leu³²–Asp³³, Leu⁵⁴–Glu⁵⁵ and Leu¹⁴³–Pro¹⁴⁴). The peptide bond Tyr²⁰–Ser²¹ is apparently scissile to trypsin in most cases because it was reported earlier by several workers on different substrates (Asao, Tsuji, Tashiro, Iwami, & Ibuki, 1992; Cheison

et al., 2010; Greene & Giordano, 1969), and attributed to trypsin and not contaminating enzymes. This cleavage was observed in all buffers except at >0.25 M potassium phosphate.

Trypsinolysis was also detected at carboxy-terminals of secondary chymotrypsinolytic bonds formed by methionine (Met¹⁴⁵–His¹⁴⁶), isoleucine (Ile²–Val³, Ile²⁹–Ser³⁰, Ile⁵⁶–Leu⁵⁷ and Ile⁸⁴–Asp⁸⁵), alanine (Ala¹⁶–Gly¹⁷ and Ala⁸⁶–Leu⁸⁷), Val (Val⁴¹–Tyr⁴²) and serine (Ser²¹–Leu²², Ser¹¹⁰–Ala¹¹¹ and Ser¹⁵⁰–Phe¹⁵¹). This shift to chymotrypsin-like activity was reported earlier in our work (Cheison et al., 2010). It was attributed to the fact that the two enzymes contain large conserved regions in their zymogens, including the catalytic triad in the active pocket (His⁵⁷, Asp¹⁸⁹, and Ser¹⁹⁵) which might implicate a primitive gene in their evolution and hence the presence of ‘chymotrypsin memory’ in trypsin. This ability of trypsin to shift to chymotrypsin-like activity was previously attributed to the production of pseudotrypsin with long storage (Keil–Dlouhá, Zylber, Imhoff, Tong, & Keil, 1971). Similar trends were observed with the hydrolysis of glucagon where crystalline trypsin also cleaved the carboxy terminals of phenylalanine and tryptophan (Keil–Dlouhá, Zylber, Tong, & Keil, 1971).

Notably, the previously reported (Cheison et al., 2010) trypsinolytic resistant peptide bond formed by Lys⁴⁷–Pro⁴⁸ was cleaved to release a non-specific peptide (f32–47) after cleavage of the Leu³¹–Leu³² bond only in NaOH–water. Additionally, fragments f(41–60) and f(92–100), situated in a core that has been considered resistant to trypsinolysis in subsequent work, were produced at all buffer concentrations except at 0.5 and 1 M potassium phosphate. It is postulated that, at high potassium phosphate concentration, the protein aggregated, leading to the hiding of some putative hydrolysis sites. Indeed, it was observed that when heat-denaturing was attempted, potassium phosphate-, and not Tris–HCl-, buffered solutions were the least stable, even at low buffer concentrations. This phenomenon likely confirms the findings that β -Lg dimerisation was favoured by an increase in the ionic strength (Vardhanabhuti & Foegeding, 2008), and influenced by protein concentration.

3.4. MALDI-TOF/MS monitoring of peptide evolution

The trypsin attack sequence on β -Lg is of great interest in our study. It is intended to determine points in the hydrolysis process when certain peptide precursors are further cleaved and eliminated (for example those peptides implicated in epitopic reactions and cytotoxicity (Hartmann, Wal, Bernard, & Pentzien, 2007)). In addition, it is intended to determine a time to stop the reaction just when particular peptides of interest having specific functionalities are released in order to prevent further cleavage and loss. The evolution of peptides over time, monitored at 5 min intervals, is shown in the 700–4000 Da range in Fig. 3. A more general trend appears in which it is easy to conclude that trypsin attacks β -Lg randomly under all conditions to initially release common peptides, such as f(1–8), f(15–40), f(41–60) and f(142–148) (results not shown). Interestingly, although it is apparent that f(149–162) is released, this peptide was not detected at any time. It was reported in our earlier work (Cheison et al., 2010) that this peptide was involved in disulphide cross-linking to either f(41–70) or f(61–70) with the resulting sizes of 5141.548 and 2847.292 Da, respectively.

From Fig. 3, it can also be seen (as expected) that the peptide peaks increased in size between 5 and 15 min but decreased by 30 min. This phenomenon has been reported by other workers (N’Negue et al., 2006; Schmelzer et al., 2007) who found that thermolysin and pepsin hydrolysed proteins to release intermediate peptides which were attacked later on. The reduction in size of the peptide peaks is, therefore accounted for by further cleavages, marked by production of smaller peptides associated with non-specific activity (results not shown). It is noteworthy, however, that some of the peptides produced initially were preserved

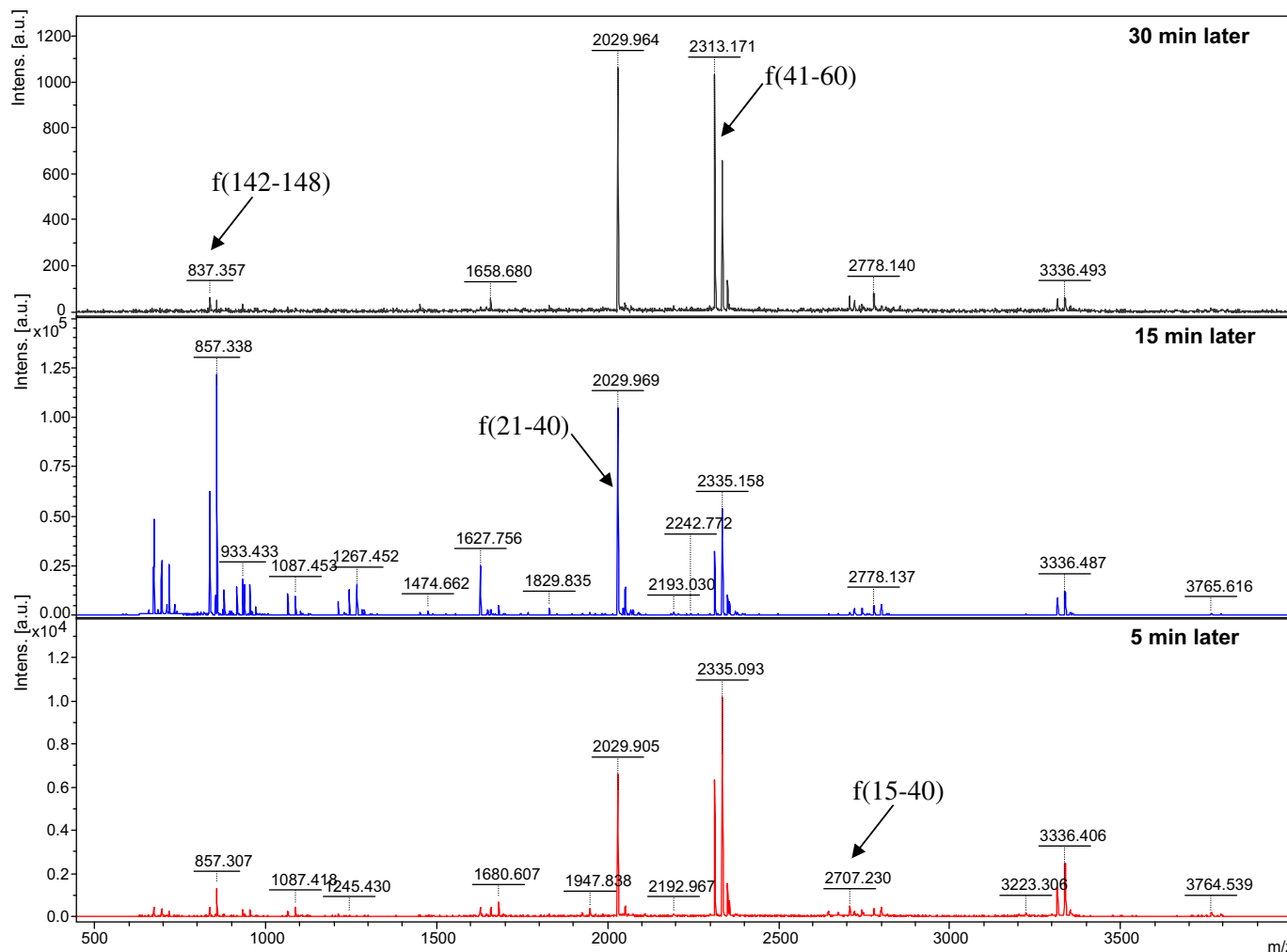


Fig. 3. A MALDI-TOF/MS chromatogram of Tris-HCl hydrolysates, showing the m/z ratio of 700–4000 Da with peptide peaks analysed at 5, 15 and 30 min. Some identified peptide signals, such as sequences $f(15-40)$, $f(21-40)$, $f(41-60)$ and $f(142-148)$, are highlighted.

throughout, even though the enzyme showed the property of hydrolysing peptide bonds not associated with trypsinolysis. The non-specific peptide signal, identified at a m/z ratio of 2029.905–2029.969, was associated with fragment $f(21-40)$ due to cleavage of Tyr²⁰-Ser²¹. It seems to be among the first products of trypsinolysis released, even though the enzyme can still access bonds designated as being scissile to trypsin. These MALDI-TOF/MS results encouraged us to introduce a stricter time regulation in a current study in which a quasi-stop-flow system was employed with peptides monitored from 5 s after the addition of trypsin. Hopefully, more insights of the sequence of attack will be gained. Benefits include the possibility of determining when to stop the process if the wanted peptides are produced, in order not to lose them. This provides potential for targeted hydrolysis, and what we call ‘look and cook’ as opposed to a ‘cook and look’ scenario, where hydrolysis is performed, followed by a search for peptides.

4. Conclusions

Hydrolysis in buffer revealed the influence of the buffering environment on the trypsinolysis of pure β -Lg and an influence on the trypsin attack patterns. Compared to NaOH-buffered water, Tris-HCl provided similar peptide compositions. Peptides produced in potassium-phosphate buffer differed from those produced in Tris-HCl at higher buffer concentrations. Monitoring of

the peptide evolution 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, non-specific cleavage of Tyr²⁰-Ser²¹ was observed to occur randomly at the initial stages of trypsinolysis. Trypsinolysis shifted from the C-terminal of lysine formed peptides to the N-terminal. Increasing potassium-phosphate buffer likely led to dimerisation and hence resistance of a core region of β -Lg. This hydrolysis medium change offered the possibility of using reduced amounts of buffer salts while producing a variety of hydrolysates at low to high buffer concentrations. Manipulating the hydrolysis environment holds potential for producing preferable peptides associated with those conditions while obviating peptides that are unwanted for one reason or another.

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