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Analysis of the Effect of Temperature Changes Combined with Different Alkaline pH on the β-Lactoglobulin Trypsin Hydrolysis Pattern Using MALDI-TOF-MS/MS

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ABSTRACT: Temperature and pH influence the conformation of the whey protein β-lactoglobulin (β-Lg) monomer, dimer, and octamer formation, its denaturation, and solubility. Most hydrolyses have been reported at trypsin (EC 3.4.21.4) optimum conditions (pH 7.8 and 37 °C), while the hydrolysate mass spectrometry was largely limited to peptides with <4 kDa. There are few reports on trypsin peptide release patterns away from optimum. This work investigated the influence of alkaline (8.65 and 9.5) and optimum (7.8) pH at different temperatures (25, 37.5, and 50 °C) on β-Lg (7.5%, w/v) hydrolysis. Sample aliquots were drawn out before the addition of trypsin (blank sample) and at various time intervals (15 s to 10 min) thereafter. Matrix-assisted laser desorption/ionization time-of-flight tandem mass spectrometry (MALDI-TOF-MS/MS) was used to monitor peptide evolution over time with the use of two matrixes: α-cyano-4-hydroxycinnamic acid (HCCA) and 2.5-dihydroxyacetophenone (DHAP). Mass analysis showed that the N- and C-terminals (Lys_8-Gly_9, Lys_{100}-Lys_{101}, Arg_{124}-Thr_{125}, Lys_{141}-Ala_{142}, and Arg_{148}-Leu_{149}) of β-Lg were cleaved early (15 s) implying the ease of trypsinolysis at the exposed terminals. Hydrolyses at 25 °C and pH 7.8 as well as at 50 °C and pH 9.5 were slowed down and ordered. Nonspecific chymotrypsin-like behavior occurred more at higher temperatures (50 °C) than at lower ones (25 and 37.5 °C). In addition to our earlier work in the acid pH region, it can be concluded that there is potential for controlled hydrolysis outside the trypsin optimum, where different target peptides with predictable biofunctionalities could be produced.

KEYWORDS: Trypsin hydrolysis, β-lactoglobulin, alkaline pH, MALDI-TOF-MS(/MS)

INTRODUCTION

The amount of new dairy products with bioactive peptides that are launched in the market shows a growing trend toward the wish of consumers to have products with additional benefits. Whey protein peptides bearing this biological activity are released by enzymatic hydrolysis. However, there is still no information about the influence of environmental factors on its hydrolysis pattern. β-lactoglobulin (β-Lg) is the major whey protein found in the milk of most mammals (∼10% of total protein or ∼50% of whey protein).[1] It is a small, soluble, and globular protein with 162 amino acids and a monomer molecular weight of 18.3 kDa.[2] There are 13 different genetic variants that are currently known.[12] The most common ones are β-Lg A and β-Lg B (Figure 1). The structure of native β-Lg is well known from X-ray crystallographic and high resolution nuclear magnetic resonance (NMR) studies. The secondary structure consists of about 6−10% α-helix, 44−52% β-sheet, 8−10% turn, and 32−35% of random coil.[3,4] These structures form nine strands of antiparallel β-sheet, eight of which form a hydrophobic barrel that is bordered by an α-helix on one side.[5,6] Bovine β-Lg has five Cys residues; four of them form disulfide bridges between Cys_{66} and Cys_{160}, and between Cys_{106} and Cys_{119}. Cys_{121} is free and therefore available for reaction.

Temperature and pH influence the conformation of the whey protein β-Lg monomer, dimer, and octamer formation, its denaturation, and solubility. The three-dimensional structure of β-Lg is also influenced by buffer ionic strength.[7−9] Tanford et al.[9] observed that a general conformational change 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 a change of the environment of a tyrosine moiety that leads to its exposure. Thus, 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 that once it is altered, it is difficult to regain. 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.[9−12]

The molecular structure of β-Lg strongly depends on the pH.[13] Under physiological conditions, it exists as a dimer but dissociates at pH between 2 and 3 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

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Figure 1. Amino acid sequence of bovine β-Lg (variants A and B). The cleavage sites for trypsin (Lys and Arg) are highlighted.

results in the denaturation of β-Lg. Generally, alkaline denaturation of bovine β-Lg becomes significant above pH 8 and increases with pH increase from 8 to 9. With pH increase above 10, further structural changes occur until at pH 12 the structure is completely random.[12,14]

Early work on bovine β-Lg suggested that the dimer dissociation occurs between 30 and 55 °C.[15] Bovine trypsin (EC 3.4.21.4), is often used in the food industry because it has high cleavage specificity, has high activity, and is very stable under different conditions.[16] Trypsin, like chymotrypsin, is a member of the serine protease S1 family. They share the catalytic triad (His_{57}, Asp_{102}, and Ser_{195}) in the active center and 40% conserved homology in the amino acid sequence. The preferential cleavage sites of trypsin are the carboxyl ends of Lys and Arg, except when a Pro is bonded to them.[16] The primary structure of β-Lg consists of 15 lysyl and three arginyl residues, but Lys_{47} is followed by a Pro_{48}; hence, there are 17 preferred cleavage sites in β-Lg (Figure 1) giving a maximum expected degree of hydrolysis, DH, of 10.56%.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), in which the favorite molecule is cocrystallized with a light-adsorbing compound, the matrix, has successfully been applied in peptide analysis.[17−19] To cover the huge variety of peptides resulting from an enzyme hydrolysis, different possible matrixes may be used. Generally, α-cyano-4-hydroxycinnamic acid (HCCA) is used for the measurement of peptides and proteins in the low mass range (700−4,000 Da),[19] whereas 2.5-dihydroxyacetophenone (DHAP)[20,21] is used for proteins and glycoproteins in the higher mass range. Despite its versatility as an analytical tool, MALDI-TOF-MS has limitations such as interference from buffer salts and matrix ions,[22] and many studies have largely used HCCA as a preferred single matrix for analysis. Consequently, only peptide fragments in the range 750−4,000 Da were analyzed to the exclusion of larger polypeptides and residual substrate.[19,21,23] This strategy has led to an under-reporting of the effect of enzyme hydrolysis.[24]

To date, most work with the trypsinolysis of β-Lg was done at declared optimum conditions for trypsin. Previous work in our group has shown that hydrolysis pH and temperature,[25] as well as hydrolysis under different buffer types and their concentration,[26] influenced the trypsin hydrolysis pattern in β-Lg. However, those analyses was performed using LC-ESI-TOF, which could only provide information on peptide patterns in the <4 kDa range. Little or no information exists in the literature on the total analysis of β-Lg hydrolysate composition in order to understand the cleavage pattern as influenced by the hydrolysis environment. Encouraging results with MALDI-TOF-MS/MS hydrolysates of β-Lg under acidic pH showed that trypsin attack was slow, ordered, and sequential away from pH 7.8 but random and disordered close to and at the optimum conditions.[27] The objective of the present study was to monitor the time-course evolution of β-Lg peptides using MALDI-TOF-MS, rather than an analysis of the end results. It is important to know how the milieu conditions influence the enzyme kinetics and peptide bond attack sequence in order to establish a processing platform for the avoidance or production of target peptides with specific biofunctionalities.

Hence, the effects of the different temperatures (25, 37.5, and 50 °C) in the optimum and alkaline pH (7.8 and 8.65 and 9.5, respectively) were studied. The working hypothesis in the present work was that with these conditions the protein conformation was changed; hence, the proteolytic patterns were different in comparison to those obtained when hydrolysis was performed at trypsin optimum conditions (pH 7.8 and 37 °C). Analysis of the time-course of hydrolysis and the produced peptides would make it possible to find a condition that could be used for the release of target peptides, in order to determine when to stop the process if the desired peptide were produced. This would lead to better process control and the development of peptide design processing platforms.

■ MATERIALS AND METHODS

Experimental Requirements. Bovine β-Lg was prepared from whey protein isolate from Fonterra Co-operative Group Ltd. (Auckland, New Zealand) as described by Gésan-Guiziou[28] using an optimized method. The β-Lg powder was made up of 95.9% native β-Lg, the rest being denatured β-Lg and caseinomacropeptide (CMP). With regard to the different genetic variants, the powder was composed of 59% β-Lg A and 41% β-Lg B. Trypsin (T9201, bovine pancrease with about 7500 units/mg with residual chymotrypsin activity of <4 benzoyl-L-tyrosine ethyl ester, BTEE, units/mg protein) was purchased from Sigma-Aldrich (St Lois, MO, USA). An autotitrator (pH-Stat, TitroLine alpha plus, Schott AG, Mainz, Germany) and a magnetic stirrer (IKA combimag rct, IKA Werke GmbH, Staufen, Germany) were used for the pH-stat hydrolysis. The whole process was controlled by TitriSoft 2.5 software (Schott AG, Mainz, Germany). Other chemicals and equipment are mentioned under respective methods.

Experimental Design. The experimental parameters used in this study were temperature (25, 37.5, and 50 °C) and pH (7.8, 8.65 and 9.5) with a substrate concentration of 7.5% and therefore an enzyme-to-substrate

(E/S) ratio of 1.86 (units/mg), which were randomized. First, the pH was kept at trypsin optimum (7.8) combined with the three temperatures to check the effect of this factor. Further, some pretrials (identification of DH and depletion rate of β-Lg) showed that the highest effect of a changed pH was at 50 °C. The experimental design was done with Statgraphics plus 5.0 (Statpoint Technologies, Inc. Warranton, Virginia, USA), and every combination was tested three times.

β-Lactoglobulin Hydrolysis. The hydrolysis methods were reported in earlier work.[25,26] NaOH and HCl were used to adjust the pH to the experimental value. The β-Lg solution was put into a thermostatically controlled, well stirred jacketed-beaker glass batch reactor (HLL Landgraf Laborsysteme, Langenhagen, Germany) connected to a thermostatted water bath. The desired temperature and pH were adjusted and monitored for stability. Because of possible autolysis of trypsin, 47 mg of the trypsin powder was dissolved shortly before use in 600 μL of Milli-Q (Milli-Q System, Millipore Corporation, Bedford, USA) purified water. Then it was vortex-mixed for about 10 s to ensure complete solubilization. Before the addition of the enzyme to the protein solution, the zero sample ($t = 0$) was drawn into a cuvette with 1 N HCl. Then the experiments were started with the simultaneous addition of trypsin and commencement of the TitriSoft 2.5 software. The pH was kept constant according to the pH-stat method with 1 N NaOH. The volume of the NaOH consumed was used to calculate the DH.[29] The hydrolysate samples were taken at 15 s, 30 s, 45 s, 60 s, 2.5 min, 5 min, 7.5 min, and 10 min after the commencement of hydrolysis and put into cuvettes with 1 N HCl to stop the enzyme by lowering the pH to <3.5. After that, the samples were frozen at −18 °C until analysis. All experiments were carried out in triplicate.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. Samples collected at respective time intervals were analyzed for mass composition using MALDI-TOF/MS(/MS) (ultrafleXtreme MALDI-TOF/TOF, Bruker Daltonics, Bremen, Germany) with two matrixes (HCCA and DHAP).

α-Cyano-4-hydroxycinnamic Acid Preparation. For the determination of the peptide compositions, Prespotted AnchorChip PAC 384/96 by Bruker Daltonics was used. One target plate holds 96 sample spots centered on the target and was prespotted with the HCCA matrix, 24 calibrants, and 3 × 4 autoteaching spots. The nine calibrants (1−10 femtomole, fmol, each) were Bradykinin Fragment 1−7 (757.3992 Da), Angiotensin II (1,046.5418 Da), Angiotensin I (1,296.6848 Da), Neurotensin (1,672.91700 Da), Renin Substrate (1,758.9326 Da), ACTH clip 1−17 (2,093.0862 Da), ACTH clip 18−39 (2,465.1983 Da), ACTH clip 1−24 (2,932.5879 Da), and ACTH clip 7−38 (3,657.9289 Da). The covered mass range offered by the calibration standard is ∼700−4,000 Da.

First, the frozen samples were thawed and then dissolved 1:150 in 0.1% TFA to a protein range of 20 picomole (pmol)/μL. Next, 1 μL of this solution was pipetted onto the prespotted target spots. The washing step was left out as it was found to be unnecessary in earlier work.[27] Each sample was spotted four times in a line such that if one calibration spot was damaged there would be at least one correct measurement to show that error.

2,5-Dihydroxyacetophenone Preparation. For the stainless steel target, the matrix was first prepared. Therefore, 10 mg of DHAP was dissolved in oversaturated 500 μL of acetonitrile (ACN) and 0.1% TFA in the ratio 1:1 v/v. This mixture was ultrasonicated for 10 min to achieve maximum solubility and then centrifuged (1 min, 10,000 rpm) to separate the saturated solution and the rest of the insoluble DHAP matrix. The supernatant was then used for spotting. The desired concentration of the protein solutions was 5 fmol/μL to 1 pmol/μL. The hydrolysates were dissolved 1:450 in 0.1% TFA. Next, the samples and the matrix were carefully mixed on cuvette caps in the ratio 1:1 (v/v) by pipetting up and down several times, and 1.5 μL of these mixtures were spotted on a 600 μm anchor target and dried at room temperature. Every sample was spotted four times. The external standard (Standard I, Bruker Daltonics GmbH, Bremen, Germany) was dissolved in 500 μL of the same ACN and 0.1% TFA solution that was used for the DHAP and vortex-mixed. After that, the standard was mixed 1:1 with the DHAP-matrix, and 1.5 μL of this was spotted four times in a specific pattern on the target. The standards were made up of insulin (5734.51 Da), ubiquitin I (8565.76 Da), cytochrome *c* (12360.97 Da), myoglobin (16952.30 Da), cytochrome *c* (6180.99 Da), and myoglobin (8476.65 Da) giving linear analysis in the range of 5k Da to undigested monomeric β-Lg.

Mass Spectrometry. MALDI-TOF/MS was run in the positive reflectron (RP) mode, managed using flexControl 3.0 Software (Bruker Daltonics, Bremen, Germany). For the HCCA-matrix, the flexControl 3.0 method RP-PepMix.par for peptide analysis was chosen, modified, and run in autoMS. The laser strength was optimized at 28% above threshold. Additionally, the spots were shot twice with increasing laser intensity for the MS/MS. The DHAP-matrix was analyzed with the flexControl 3.0 method RP-ProtMix.par, also run in autoMS. In this case, no MS/MS analysis was performed because it is common that only peptides up to a size of about 4,000 Da were fragmented. The optimum laser strength for this matrix was 45% above the threshold. The peaks were picked using flexAnalysis 3.0, submitted to BioTools 3.2 build 3.2.1.31 and compared to a theoretical digest done using the Sequence Editor, all from Bruker Daltonics (Bremen, Germany). The flexAnalysis software eliminates the peaks that are known to originate from the matrix or trypsin. Only those peptides which were available in three of the four spots were considered safe to assign a sequence. This was carried out with a program written using the database software Access (Microsoft Office 2007, Microsoft, Redmond, Washington, USA) which searched for triplicate matches. After that, the results of the three experiments done with the same condition were also put into this database to search for peaks appearing reproducibly in at least two of the three measurements. The sequences that fulfilled these requirements were considered to be safe. The MS/MS analysis was done in two different ways. If the concentration of the fragments or the single amino acids were high enough, the sequence could be detected by BioTools. Therefore, a theoretical digest with the Sequence editor was performed, and these theoretical fragments were sent to BioTools together with the peaks which were picked at flexAnalysis. If the program could not relate the peaks that were picked with MS/MS to a sequence automatically, this was done manually.

RESULTS AND DISCUSSION

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. For each of the chosen conditions, the time-course evolution of peptides at time $t = 0$ s, 15 s, 30 s, 45 s, 60 s, 2.5 min, 5 min, 7.5 min, and 10 min were studied in triplicate trials. To confirm that there was no autolysis at extreme pH (9.5) and temperature (50 °C), the sample for $t_{(50\ °C;\ pH 9.5)} = 0$ was analyzed with DHAP and HCCA matrixes, and the results confirmed only the presence of singly charged, doubly charged, and triply charged β-Lg ions (similar to $t = 0$ in Figure 2a and b) in the DHAP matrix chromatograms. The HCCA matrix for the smaller peptides revealed that there were no signal peaks either, which means the peaks detected in MALDI-TOF-MS during subsequent times could be assigned to enzyme hydrolysis.

Influence of the Hydrolysis Time on Peptide Evolution. The evolution of peptides over time, at 50 °C and pH 7.8, monitored at time zero ($t_{(50\ °C;\ pH 7.8)} = 0$), 30 s, and 10 min, is shown for the HCCA (800 to 4,000 Da range) and DHAP matrix analysis (5,000 Da to undigested β-Lg range) in Figure 2a and b, respectively. The HCAA matrix analysis shows that before hydrolysis ($t = 0$), there were no peptide signals in the <4,000

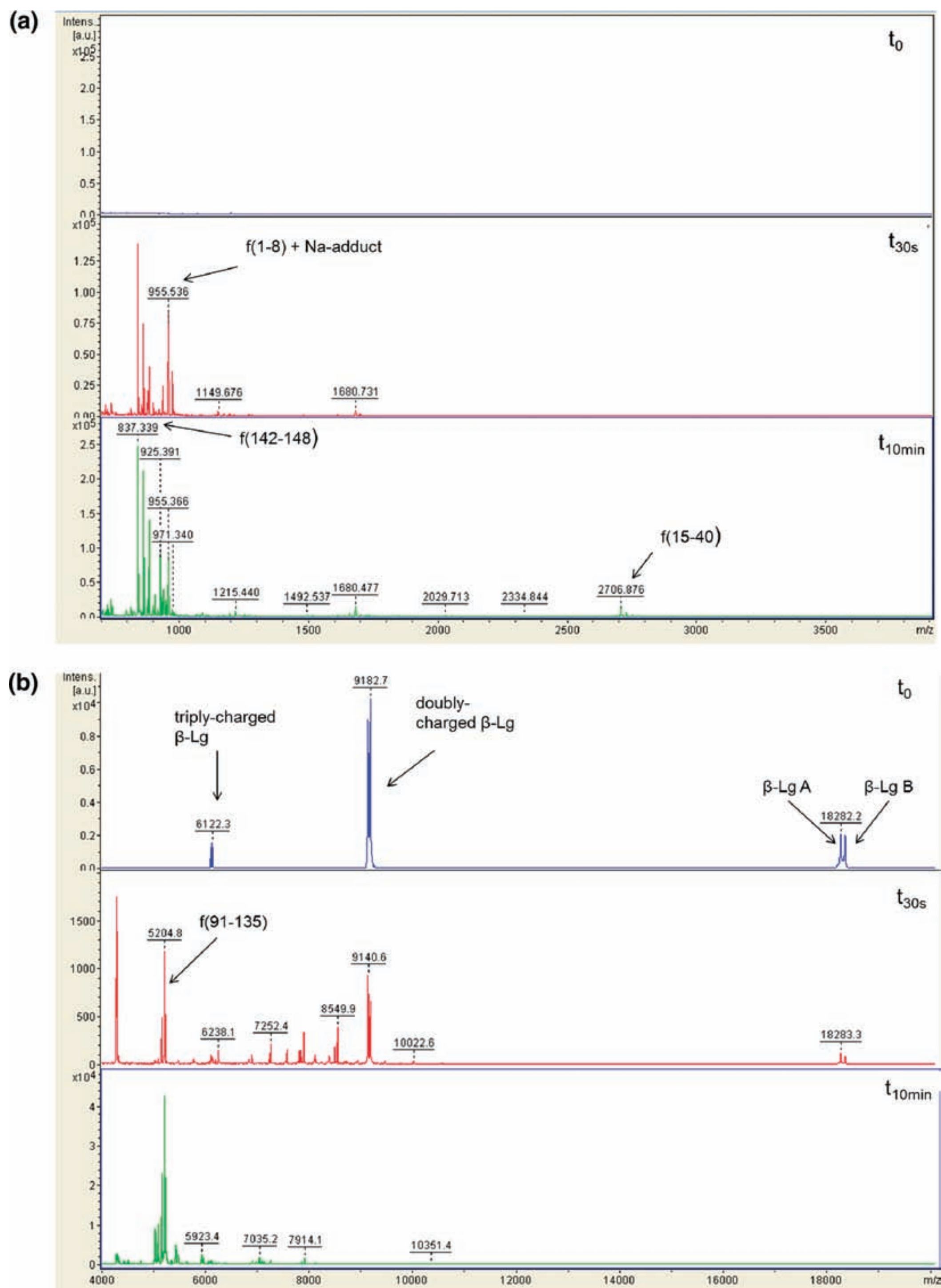

Figure 2. (a) Spectrogram of samples analyzed using the HCCA matrix at 50 °C and pH 7.8 at time t = 0, 30 s, and 10 min showing no peaks before hydrolysis (t = 0) and a sodium ion adduct (+22 m/z) on f(1−8). (b) Spectrogram of samples analyzed using the DHAP matrix at 50 °C and pH 7.8 at time t = 0, 30 s, and 10 min showing some intermediate peptides at 30 s and virtually no more β-Lg after 10 min of hydrolysis.

Da range. However, 30 s later, there were peaks for the initial attack sites, notably f(1−8) and its sodium ion adduct (+22) appearing at around 955 Da. This is a common adduct found in all hydrolysates under NaOH in our studies. Notably, the signal detected at 2,029 Da after 10 min of hydrolysis is a typical product of the cleavage of Tyr^{20}-Ser^{21} (f(21−40)) in β-Lg, a side reaction which is probably to be considered in redefining the narrow specificity assigned trypsin since it is a common feature detected in various substrates even when the purest possible trypsin was used.

With the DHAP matrix, the singly charged, doubly charged, and triply charged β-Lg ions could be seen t = 0. After 30 s, these β-Lg peaks were still visible, but most of the peptide peaks were in the range between 4,000 and 10,000 Da. After 10 min of hydrolysis, some of the peaks between 6,000 and 10,000 Da disappeared, while the β-Lg substrate was depleted. The advantage

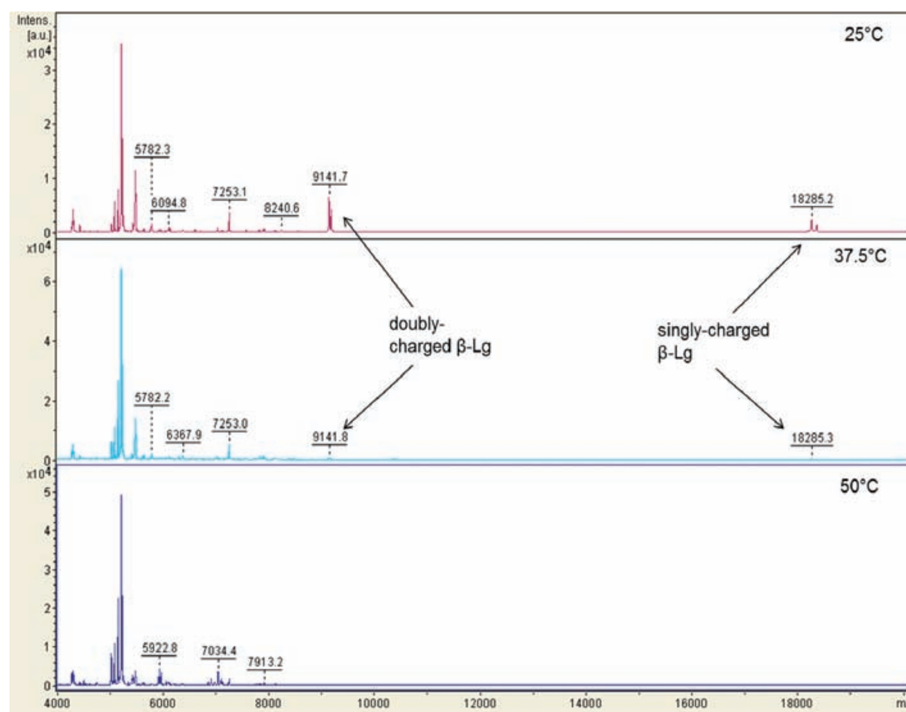

Figure 3. Spectrogram of samples analyzed using the DHAP matrix at pH 7.8 at different temperatures after 10 min of hydrolysis showing residual protein at lower temperatures.

of the two matrix system adopted in our study is clearly shown by the sum of Figure 2a and b. With this approach, it is possible to improve the acquisition of data for the total characterization of the hydrolysates. What remains, however, is the information about the hydrolysates in the <800 Da region, which is still not possible due to the noise signals from the matrixes, although recently a matrix was reported for MALDI-TOF analysis in the 0 to 1,000 Da using 1,8-bis(dimethyl-amino)naphthalene (DMAN, Proton Sponge).[30]

Influence of Temperature Change on Peptide Composition. Comparison of the HCCA matrix peaks for the samples obtained at 10 min hydrolysis at the different conditions showed that only marginal differences were caused by changes in the pH as well as an increase in temperature (data not shown). Comparison of the DHAP chromatograms for the samples produced at pH 7.8 with increasing temperatures (25, 37.5, and 50 °C) at the end of the hydrolysis (10 min) shows remarkable influence on the attack patterns (Figure 3). At 25 and 37.5 °C, singly charged and doubly charged ions of β-Lg confirm some resistance to hydrolysis at this condition, or that the enzyme attacked precursor peptides to produce shorter peptides even though β-Lg could still be detected. In contrast, at 50 °C the spectrogram reveals that the native substrate was already depleted, a feature which was observed in our earlier results with β-Lg hydrolysis at different temperatures. As is the case with MALDI-TOF so far, only qualitative data is presented because the intensity of the peaks was not a measure of the quantity of the substance responsible for that signal.

Influence of pH Change on Peptide Composition. The chromatograms of the DHAP matrix illustrating the influence of the pH after 10 min hydrolysis at 50 °C can be seen in Figure 4. These results show that at pH 7.8 and 8.65 there were quite similar distributions of the resulting peptides, whereas at pH 9.5 an absolutely different pattern was obtained. At pH 9.5, the peaks with m/z of 5,000 to 5,600 were almost not detectable, but residual β-Lg was detected as can be confirmed by the multiply charged ion peaks.

The slow digestion of β-Lg that was seen at 50 °C and pH 9.5 was caused by the loss of activity of the enzyme over time under this condition and protein inhibition, which was observed when denatured whey protein isolate was hydrolyzed using various enzymes by Cheison et al.[31] Therefore, if the peptides of this range are of interest, this condition could be used to protect the peptides because at the other conditions, those peptides were depleted. However, if residual β-Lg were considered a nuisance, for example, in allergy, then this condition should be avoided for limited hydrolysis of β-Lg. Generally, there were no peaks found with a mass between 11,000 Da and about 18,000 Da (β-Lg). This leads to the conclusion, that even at the first sampling (15 s), the protein was already cleaved more than once with the resulting peptides being <10 kDa. On the basis of the calibration standards and matrixes used, the peaks with m/z ratios larger than 800 Da were considered in order to exclude the background noise or ionized matrix. An estimation of the loss that is caused therefore could be done according to Galvão et al.[32] who determined that about 40% of the peptides were smaller than 750 Da after hydrolysis to the maximum possible DH (12% for trypsin). Hence, it would not be possible to detect them with the matrixes used in this work. Hydrolysis for 10 min may not have yielded such a high proportion of smaller peptides, however.

Influence of the Temperature on the Qualitative Proportion of Peptides. A combination of the information on the number of peptides detected during hydrolysis could be achieved with the sum of the peptides detected under the two matrixes used in this study. For example, the effect of an increase in the temperature at pH 7.8 after 15 s, 5 min, and 10 min is shown in Figure 5. It can be seen that at 25 and 37.5 °C only short peptides were detected after 15 s, apart from one peptide in the range of

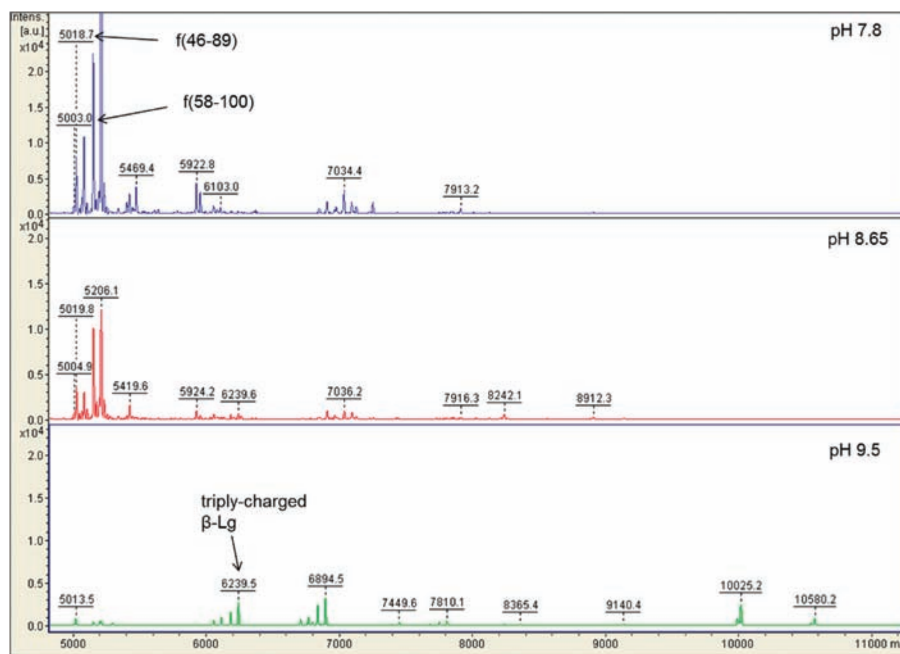

Figure 4. Spectrogram of samples analyzed using the DHAP matrix at 50 °C with increasing pH values after 10 min of hydrolysis showing the presence of undigested β-Lg at pH 9.5.

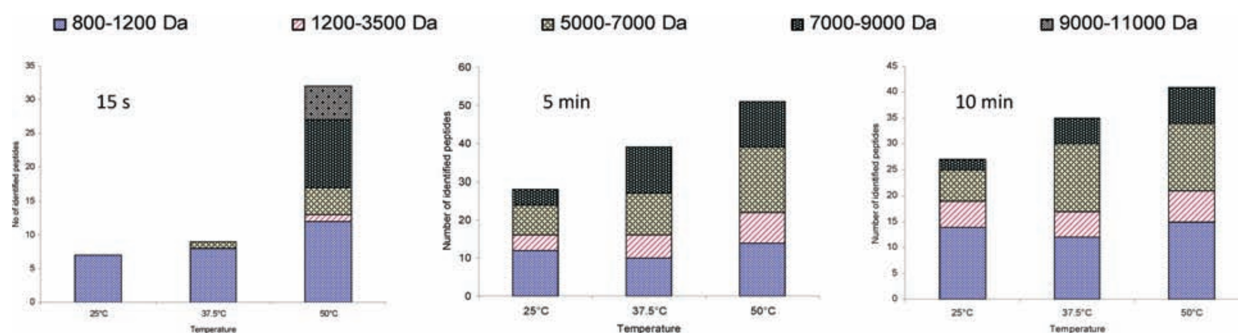

Figure 5. Qualitative proportion of the specific peptides evolved over time at pH 7.8 and different temperatures showing peptides lumped into size ranges.

5,000−7,000 Da at 37.5 °C. There were also larger fragments which were not detected. This may be caused by ordered cleavage of the terminal parts that resulted in specific peptides that could be determined and a further random cleavage of the bigger fragments caused by the determination of the m/z that were only seen to be true when they appeared in at least two of the triplicates, the larger fragments were perhaps excluded because their appearance was not reproducible.

At 50 °C, many small and large peptides were detected, with fewer fragments in the medium range. After 5 min of hydrolysis, there were many more peptides than at 15 s, whereas the number of different peptides increased with increasing temperature. After 10 min, a similar distribution to that observed after 5 min was noticed, but altogether there were fewer peptides. This loss in the variety of peptides obtained after 10 min of hydrolysis is obviously caused by a further cleavage of the precursor peptides, resulting in nonspecific peptides such as f(21−40) shown in Figure 2.

Influence of pH on Proportion of Peptides. A similar treatment of data to ascertain the influence of pH on peptide distribution showed a trend similar to that observed in Figure 5. Generally, at the beginning there were mostly small or large peptides. The variety of peptides between 1,200 and 3,500 Da was low at every pH. Later during hydrolysis, at pH 7.8 and 8.65, the large fragments between 9,000 Da and 11,000 Da were cleaved to shorter peptides. The variety of peptides in the mass range of 5,000 Da to 7,000 Da increased the most. The small peptides between 800 Da to 1,200 Da were more prevalent at pH 7.8 than at the other two pH values. At pH 9.5, the peptides in the mass range between 9,000 and 11,000 Da were not cleaved further, as was the case at the lower pH values of 8.65 or 7.8. This could be because of the slow hydrolysis under this condition. Regarding the time-course evolution of the 5,000−7,000 Da fragments at pH 9.5, results show that they increased from 15 s to 5 min, with little changes between 5 and 10 min.

Analysis of Cleavage Sites. *Specific Cleavage.* A further interesting point to characterize the β-Lg cleavage behavior of trypsin under different conditions is the specific cleavage sites, the carboxy-terminals of Lys and Arg. Some of the specific peptides became undetected over time due to further cleavage into smaller peptides and the fact that those peptides were below the matrix range (<800 Da). The specific cleavage bonds that were likely to be hydrolyzed by trypsin are highlighted in Figure 1.

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Table 1. Initial Cleavage Sites under the Different Conditions Monitored at 15 s

	25 °C, pH 7.8	37.5 °C, pH 7.8	50 °C, pH 7.8	50 °C, pH 8.65	50 °C, pH 9.5
Lys_8	x	x	x	x	x
Lys_{14}					
Arg_{40}					
Lys_{47}					
Lys_{60}			x		
Lys_{69}					
Lys_{70}					
Lys_{75}			x		
Lys_{77}			x		
Lys_{83}					
Lys_{91}		x			x
Lys_{100}	x	x	x	x	x
Lys_{101}		x	x		
Arg_{124}	x	x	x	x	x
Lys_{135}				x	x
Lys_{138}		x	x		
Lys_{141}	x	x	x	x	x
Arg_{148}	x	x	x	x	x

The bond (Lys_{47}-Pro_{48}) is reportedly resistant and was unlikely to be cleaved during hydrolysis with trypsin because of the imino acid kink-like bond structure formed by Pro.[16,25]

Initial Cleavage. As shown in Table 1, the C-terminal cleavage sites of Lys_8, Lys_{100}, Arg_{124}, Lys_{141}, and Arg_{148} were attacked at the beginning of every hydrolysis condition. This can be explained by the β-Lg structure because all of these are bonds that are located at the N- and C-terminals which are exposed and accessible to the enzyme at the beginning of the reaction. Trypsinolysis of β-Lg under acid conditions showed that the peptide bonds Lys_8-Gly_9, Lys_{141}-Ala_{142}, and Arg_{148}-Leu_{149} were initial cleavage sites.[27] At the optimum condition of 37.5 °C and pH 7.8, eight bonds were cleaved in the first 15 s, while the highest initial peptide bond cleavage was at 50 °C and pH 7.8 with 10 bonds cleaved. The higher initial cleavage at 50 °C and pH 7.8 could be explained by the high rate of β-Lg depletion at 5 min (v_5) under this condition. Additionally, it could be that the higher temperature caused structural changes that made the protein better accessible for the enzyme in addition to the working pH which is optimum (pH 7.8) for trypsin. This could also explain the cleavage in the center of β-Lg (Lys_{60}, Lys_{75}, and Lys_{77}), which was designated as a resistant core in our earlier work.[27]

Influence of the Temperature on Specific Cleavage. Table 2 shows the cleavage of the trypsin-specific bonds in the initial (15 s) and at the end of the 10 min hydrolysis at pH 7.8. This reflects the results of the DH_{10} in which the percentage of the total bonds that were cleaved after 10 min increased from 25 °C over 37.5 to 50 °C. At 10 min under pH 7.8 and 50 °C, all possible specific bonds were cleaved. Further, Lys_{138} was only cleaved at pH 7.8 and 50 °C, but this is obviously based on the further cleavage of the fragment f(139−148) to f(139−141) and f(142−148). However, the fragment f(139−141) was too small to detect (~300 Da) with the matrixes used in this work, although it was reported in our earlier work analyzed using LC-ESI-TOF-MS.[25] At 25 °C, only the terminal regions of β-Lg were cleaved with the core of the protein showing resistance. However, with increasing temperature, the protein core was hydrolyzed. Throughout this work, Lys_{47}-Pro_{48} was undigested, confirming Pro influence on bond resistance to trypsin.

Table 2. Cleavage Sites at pH 7.8 at Different Temperatures (Time: 15 s and 10 min)

	25 °C		37.5 °C		50 °C	
	15 s	10 min	15 s	10 min	15 s	10 min
Lys_8	x	x	x	x	x	x
Lys_{14}		x		x		x
Arg_{40}		x		x		x
Lys_{47}						
Lys_{60}				x	x	x
Lys_{69}		x		x		x
Lys_{70}				x		x
Lys_{75}		x		x	x	x
Lys_{77}		x		x	x	x
Lys_{83}		x		x		x
Lys_{91}		x	x	x		x
Lys_{100}	x	x	x	x	x	x
Lys_{101}		x	x	x	x	x
Arg_{124}	x	x	x	x	x	x
Lys_{135}		x		x		x
Lys_{138}			x		x	x
Lys_{141}	x	x	x	x	x	x
Arg_{148}	x	x	x	x	x	x

Influence of the pH on Specific Cleavage. The influence of pH on the specific hydrolysis pattern (results not shown) at 50 °C at the initial (15 s) and final cleavage (10 min) showed that bonds Lys_{60}, Lys_{75}, and Lys_{77} were cleaved. It was observed that at 50 °C there was a v_5 decrease with increasing pH. This explains why bonds cleaved after 15 s at pH 7.8 were hydrolyzed later at pH 8.65 and 9.5. Further, it was observed that some bonds were resistant to trypsin cleavage under pH 8.65 (Lys_{91}−Val_{92}, Lys_{138}−Ala_{139}) and pH 9.5 (Lys_{70}−Ile_{71}). The bond Lys_{101}−

Table 3. Analysis of the Time-Course Cleavage of Peptide Bonds at Optimum Conditions (37.5°C and pH 7.8)

	0 s	15 s	30 s	45 s	60 s	2.5 min	5 min	7.5 min	10 min
Lys_{8}		x	x	x	x	x	x	x	x
Lys_{14}			x	x	x	x	x	x	x
Arg_{40}			x	x	x	x	x	x	x
Lys_{47}									
Lys_{60}			x	x	x	x	x	x	x
Lys_{69}					x	x	x	x	x
Lys_{70}								x	x
Lys_{75}					x	x	x	x	x
Lys_{77}			x	x	x	x			
Lys_{83}					x	x	x	x	x
Lys_{91}		x	x	x	x	x	x	x	x
Lys_{100}		x	x	x	x	x	x	x	x
Lys_{101}		x	x	x	x	x	x	x	x
Arg_{124}		x	x	x	x	x	x	x	x
Lys_{135}			x	x	x	x	x	x	x
Lys_{138}		x	x	x	x	x			
Lys_{141}		x	x	x	x	x	x	x	x
Arg_{148}		x	x	x	x	x	x	x	x

Tyr_{102} was resistant at 50 °C at higher pH values of 8.65 and pH 9.5, whereas it was hydrolyzed under every other condition used (pH 7.8 with 25, 37.5, and 50 °C). Therefore, it seems that the alkaline pH changes the structure of β-Lg and/or trypsin in such a way that it influences bond cleavage. First, the combination of high temperature (50 °C) and high pH values (8.65, 9.5) probably caused denaturation resulting in β-Lg structure changes. At pH 9.5, this can additionally be due to the influence of the Tyr residue at the C-terminal side, which begins to deprotonate in this pH region, and therefore, the susceptibility to trypsin may be affected. These results confirm the influence of the changing milieu conditions on the hydrolysis pattern, and it is hoped that the failure to hydrolyze some of the peptide bonds under these conditions may be used to protect precursor peptides and steer hydrolysis toward a certain predicted objective.

Influence of the Hydrolysis Time on the Specific Cleavage. The time-course β-Lg trypsinolysis under optimum conditions (37.5 °C, pH 7.8) was used as a reference (Table 3). It seems that the cleavage of bonds Lys_{77}-Ile_{78} and Lys_{138}-Ala_{139} did not occur after 2.5 min, although some fragments were further broken down, and the resulting peptides could not be measured because of their mass range (smaller than 800 Da). In the case of cleavage at Lys_{138}, fragment f(139−148) was broken down further into the fragments f(142−148) and f(139−141). The comparison of the results of cleavage patterns over time at trypsin optimum with those of the cleavage at 25 °C and pH 7.8 (data not shown) illustrates that many of the bonds were hydrolyzed later or not at all at 25 °C and pH 7.8 (compared with Table 2). The bonds Lys_{60}-Trp_{61} and Lys_{70}-Ile_{71}, for example, were not cleaved in the 10 min of hydrolysis at 25 °C and pH 7.8, whereas Lys_{75}-Thr_{76}, Lys_{77}-Ile_{78}, Lys_{83}-Ile_{84}, Lys_{91}-Val_{92}, Lys_{101}-Tyr_{102}, Lys_{135}-Phe_{136}, and Lys_{138}-Ala_{139} were cleaved later in comparison to the optimum conditions (37.5 °C and pH 7.8). Again, these results point to the importance of the environmental conditions in the control of the hydrolysis pattern of β-Lg by trypsin which could be used to obtain certain specific peptide patterns.

In the case of 50 °C and pH 7.8 (data not shown), the Lys_{69}-Lys_{70} and Lys_{75}-Thr_{76} bonds were cleaved earlier in the hydrolysis, whereas Lys_{83}-Ile_{84} and Lys_{91}-Val_{92} bonds were cleaved earlier under optimum conditions. In the case of Lys_{83}-Ile_{84}, this difference was notably high with an appearance much later at 7.5 min at 50 °C and pH 7.8 versus 60 s at optimal conditions. The fragments that were produced due to the cleavage of Lys_{77}-Ile_{78} were not cleaved further until toward the end of hydrolysis (10 min) at 50 °C and pH 7.8, similar to what happened at the optimum conditions. The cleavage at 50 °C and pH 8.65 (data not shown) showed that the bonds Lys_{91}-Val_{92} and Lys_{101}-Tyr_{102} were resistant throughout the 10 min hydrolysis. The bonds that were cleaved earlier at 50 °C and pH 7.8 (Lys_{69}-Lys_{70} and Lys_{75}-Thr_{76}) were also cleaved faster at pH 8.65. The fragments that were released due to the cleavage of Lys_{77}-Ile_{78} were detected after 2.5 min of hydrolysis and were not hydrolyzed further; they were still detectable at 10 min (under optimum conditions, further cleavage of this bond was found from 30 s to 2.5 min). The other cleavage bond that disappeared under optimal condition is Lys_{138}-Ala_{139}. At 50 °C and pH 8.65, this peptide bond was cleaved at 30 s; afterward, further cleavage appeared to be equally fast. Uniquely, at 50 °C and pH 8.65, the peptide f(94−100) that was detected due to the cleavage of Lys_{100}-Lys_{101} was also broken down after 45 s of hydrolysis.

The faster cleavage (compared to optimum conditions) of the two bonds Lys_{69}-Lys_{70} and Lys_{75}-Thr_{76} (to yield f(70−126) and f(76−83)) that was seen at 50 °C at pH 7.8 and 8.65 could not be detected when the pH was increased to 9.5. Apparently, hydrolysis at these extreme conditions (50 °C with pH 9.5) was slowed down, and this could be confirmed with the time-course hydrolysis patterns (data not shown). Additionally, this could also be seen in further cleavage of the fragments f(78−122) and f(139−148) that gives information about the cleavage of the bonds Lys_{77}-Ile_{78} and Lys_{138}-Ala_{139}. Under optimum conditions, both were further degraded, but at 50 °C and pH 9.5, they did not appear until 7.5 min for Lys_{77}-Ile_{78} (f(78−122)) and 10 min for Lys_{138}-Ala_{139} (f(139−148)), and therefore if there was any degradation under these conditions, it could not be seen in the hydrolysis time (10 min). Under this condition, Lys_{101}-Tyr_{102} was probably not cleaved because the fragment f(92−101) did not appear, implying resistance up to 10 min hydrolysis. Obviously, if this were the target for conservation, a choice of this condition could be used to steer the enzyme attack in order to achieve that objective.

There were no differences in the cleavage time of Arg_{124}-Thr_{125} and Arg_{148}-Leu_{149} and nearly no difference at Arg_{40}-Val_{41} at all hydrolysis conditions. Therefore, the cleavage behind Arg seems to be less affected by the change in the hydrolysis conditions. Argynyl bonds were reported to be 25-fold more scissile to trypsin than lysyl bonds,[33] and this may explain their apparent ease of hydrolysis.

Nonspecific Cleavage of β-Lactoglobulin. It is generally accepted that trypsin releases peptides due to the cleavage of peptide bonds formed by Lys and Arg bonds and any other amino acid on the C-terminal, except when the other amino acid is a Pro. However, peptides were also reported to be released by the cleavage of peptide bonds other than by cleavage of the specific sites. A shift from the trypsinolysis of the carboxy- to the N-terminal end of Lys was noted earlier in our group,[25,26] and the property was called semitrypsinolysis. In this work, this phenomenon was also detected with the release of sequence f(124−131) involving the cleavage of the N-terminal of Arg_{124} (Val_{123}-Arg_{124}). This

Table 4. Typical Chymotrypsin-Like Cleavage at the Different Conditions Independent of Their Appearance Time

	25 °C, pH 7.8	37.5 °C, pH 7.8	50 °C, pH 7.8	50 °C, pH 8.65	50 °C, pH 9.5
f(43−49)			x	x	x
f(21−30)			x	x	
f(18−28)	x	x	x		
f(11−21)			x		
f(119−133)					x

peptide was released at pH 7.8 with all temperatures (25, 37.5, and 50 °C) but was not detected at 50 °C at higher pH values, 8.65 or 9.5. However, under these alkaline conditions the sequence f(92−99) was detected following the N-terminal cleavage of Lys_{100} (Thr_{99}-Lys_{100}). Another peptide released due to the cleavage at the N-terminal site of Lys (Gln_{13}−Lys_{14}), f(14−20), was produced only at 25 °C and pH 7.8.

The trypsin (Sigma Aldrich product T8003) used in these experiments had a declared chymotrypsin activity of <4 BTEE units/mg protein. Therefore, chymotrypsin-like cleavage was not unexpected. However, when compared to the close to 10,000 BAEE units/mg protein of trypsin, it was expected that the chymotrypsin side reaction could have only minimal effects. In addition, trypsin has the tendency to change into chymotrypsin-like behavior, expressed in nonspecific cleavage. Chymotrypsin, like trypsin, is a serine proteinase of the digestive tract. It catalyzes the hydrolysis of peptide bonds on the C-terminal side of Tyr, Phe, and Trp, which all contain an aromatic ring that fit into the hydrophobic pocket.[34] In addition, it hydrolyses the C-terminal side of Leu. Additionally, secondary hydrolysis of chymotrypsin occurs on the C-terminal side of Met, Ile, Ser, Thr, Val, His, Gly, and Ala. The fragments which were released by trypsin due to typical chymotrypsin-like hydrolysis were f(43−49), f(21−30), f(18−28), f(11−21), and f(119−133). The associated cleaved bonds are Leu_{10}-Asp_{11}, Gly_{17}-Thr_{18}, Ser_{21}-Leu_{22}, Ser_{30}-Leu_{31}, Tyr_{42}-Val_{43}, Thr_{49}-Pro_{50}, Val_{118}-Cys_{119}, and Leu_{133}-Glu_{134}, respectively. The release of the different fragments, caused by chymotrypsin-like behavior, can be seen in Table 4. The fragment f(43−49) was released at 50 °C at every pH, while f(21−30) was detected throughout except at pH 9.5. The uniqueness of fragment f(43−49) is that trypsin cleaved the N-terminal of a Lys_{47} bonded to a Pro_{48} residue, which was not detected for specific cleavage. Why trypsin could hydrolyze Leu_{46}-Lys_{47} and not Lys_{47}-Pro_{48} is not easy to answer except when the chymotrypsin-like activity is considered. Fragment f(18−28) was detected at pH 7.8 at all hydrolysis temperatures. Whenever these peptides were released, they were detected between 2.5 to 7.5 min, and afterward they disappeared again, caused by further cleavage. Furthermore, they were released even when some trypsin scissile bonds were still uncleaved.

The release of the fragments f(43−49) and f(18−28) was strongly correlated to the reaction condition. Fragment f(43−49) was just released at 50 °C at different pH values, and fragment f(18−28) was released at pH 7.8 independent of the temperature. There was only one peptide (f(18−28)) that was released by the nonspecific activity at 25 and 37.5 °C, while most of them were released at 50 °C and pH 7.8. The findings that chymotrypsin-like cleavage occur more at higher temperatures, agree with earlier reports.[25] The typical shift from trypsin to chymotrypsin-like activity was attributed to the substantial identity of trypsin and chymotrypsin, in which the catalytic triad (His_{57}, Asp_{102}, and Ser_{195}) is conserved. Furthermore, trypsin and chymotrypsin share about 40% homology in the amino acid sequences. The property was attributed to the likelihood of the zymogen sharing of a primitive gene in their evolution with chymotrypsin memory being conserved in trypsin. Hence, at temperatures above 40 °C (50 °C in the current work), it was postulated that the catalytic pocket underwent a conformational change that allowed the entry and processing of bulky amino acids in the catalytic pocket, which are usually restricted by the tightness.[25]

Typically, the peptide bond Tyr_{20}-Ser_{21} (resulting in f(21−30) with mass around 965 Da) is apparently scissile to trypsin in most cases because it was reported earlier by several workers on different substrates[25,26,35,36] including during hydrolysis under pressure,[37] which were attributed to trypsin and not contaminating enzymes. Obviously, if this peptide bond were considered important not to cleave hydrolysis at 25 or 37.5 °C with pH 7.8, then 50 °C with pH 9.5 could be used.

Conclusions. Work on the specific influence of the hydrolysis environment on the trypsinolytic products were investigated and the time-line evolution of peptides analyzed with sensitive two matrix MALDI-TOF-MS(/MS). Two matrix analysis in MALDI-TOF-MS(/MS) analysis revealed that the N- and C-terminal (Lys_8-Gly_9, Lys_{100}-Lys_{101}, Arg_{124}-Thr_{125}, Lys_{141}-Ala_{142}, and Arg_{148}-Leu_{149}) peptide bonds were easily cleaved at all hydrolysis conditions within the first 15 s implying the ease of trypsinolysis. Cleavage of arginyl residues was generally faster than that for lysyl residues throughout. Nonspecific cleavage of trypsin revealed a typical chymotrypsin-like activity with the cleavage of Tyr_{20}-Ser_{21}, a feature which was reported in many studies working with pure trypsin. 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}.

Two matrixes offered the complementarity not available with single matrix analysis enabling a better understanding of the enzyme breakdown to yield both large and intermediate peptides. It remains to be seen whether a three matrix system or a combination of LC-ESI-(TOF)-MS(/MS) and MALDI-(TOF)-MS(/MS) could offer a strategy for complete hydrolysate analysis. These results offer the potential for the steering of the enzyme toward the cleavage of specific target bonds and/or avoidance of specific bonds. In this way, there exists the possibility to influence the peptide compositions through peptide design with 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 were produced in order to protect them from further cleavage.

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