



Regular article

 β -Lactoglobulin tryptic digestion: A model approach for peptide release

Ayoa Fernández, Francisco Riera*

Department of Chemical Engineering and Environmental Technology, University of Oviedo, Julián Clavería 8, 33006 Oviedo, Spain

ARTICLE INFO

Article history:

Received 31 July 2012

Received in revised form

18 September 2012

Accepted 3 October 2012

Available online xxx

Keywords:

Enzyme technology

Proteolysis

Modeling

Kinetic parameters

 β -Lactoglobulin

Trypsin

ABSTRACT

β -lg tryptic digestion leads to the release of a broad range of biologically active peptides. Understanding how these peptides are formed and the dynamics of the hydrolysis is of major relevance in order to control the quality of the final products. In this paper, the time-dependent release of peptides is reported and the dynamics of this release is discussed in terms of the physical accessibility of the enzyme to the scissile bonds and the secondary specificity of trypsin. In view of experimental data, a kinetic model based on first-order equations is proposed to simulate both β -lg degradation and peptide formation. The statistical data obtained seem to support the hypothesis that the hydrolysis mainly takes place via the proposed model. The results evaluated in this paper show the existence of areas within the intact protein with different susceptibility to tryptic attack. Whereas C- and N-terminal areas are easily digested, the internal part of the protein shows more resistance to hydrolysis and the release of final peptides within this region passes through the formation and subsequent degradation of intermediate peptides.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Whey proteins, which represent up to 20% of total protein content of milk, are one of the most widely studied sources of bioactive peptides: i.e., specific protein fragments that have a positive impact on body functions or conditions [1]. These peptides are inactive within the sequence of the parent protein and are usually released by enzyme digestion or during microbial fermentation of proteins. In the case of the main cheese whey protein, β -lactoglobulin (β -lg), its trypsin digest (EC 3.4.21.4) leads to the release of a broad range of biologically active sequences [2] as well as the production of hypoallergenic whey protein hydrolysates [3]. Furthermore, β -lg hydrolysates can also be used for a variety of technological applications due to their emulsifying, foaming and gelation properties [4,5]. Some of the aforementioned properties depend on the nature of the peptides produced and the degree of hydrolysis (DH). Hence, the understanding of peptide formation and the dynamics of the hydrolysis is of major relevance in order to control the quality of the final products.

Proteolysis has usually been described by analyzing the overall rate of hydrolysis expressed as the DH, a single variable which includes the concentration of the intact protein and the released peptides. Although this kind of approach affords useful information on the hydrolysis, it does not provide any information on the products. It should be noted that enzymatic hydrolysis reactions such as protein hydrolysis are highly complex due to the

diversity of reaction components, the multiplicity of reaction types, the complexity of reaction networks and the influence of hydrolysis conditions such as pH or temperature. It is therefore very difficult to obtain a kinetic model flexible enough to fit experimental data yet, at the same time, simple enough to allow the prediction of peptide concentration via easily identifiable parameters [6].

In the specific case of β -lg tryptic digestion, some research studies have focused on substrate degradation and steady-state kinetics [7], and some have even monitored peptide formation throughout the hydrolysis process [8]. However, to the best of our knowledge, no study has been published describing the dynamics of peptides as a kinetic model in which the concentration of each peptide is expressed as a function of hydrolysis time.

The main objective of the present paper is to propose a kinetic model to explain the dynamic formation and degradation of β -lg derived peptides obtained through trypsin digestion of β -lg. The quality of the resulting model will be assessed by comparing its output with the experimental data obtained. In addition, the study of released peptides will provide information on how a specific peptide bond is protected against trypsin action depending on its neighboring amino acids and physical accessibility to the peptide bond.

2. Material and methods

2.1. Hydrolysis experiments

Enzymatic hydrolysis experiments were performed in a 200 mL batch reactor equipped with temperature and pH control. The substrate solution was prepared by dissolving a given amount of bovine

* Corresponding author. Tel.: +34 985103436; fax: +34 985103434.

E-mail address: far@uniovi.es (F. Riera).

β -lg (Davisco Foods Co., USA) in distilled water at 37 °C. Once the protein was completely dissolved, the pH of the solution was adjusted to 8.0 and the reaction was initiated with the addition of TPCK-treated trypsin (T1426-1G, Sigma, USA). A 0.5 M NaOH solution was used to maintain the pH of the mixture at a constant value. The DH, expressed as the ratio between the amount of peptide bonds hydrolyzed during the reaction and the total amount of peptide bonds in the native protein, was calculated from the amount of base used in the pH-stat technique [9] as shown in Eq. (1):

$$\text{DH}(\%) = \frac{B \cdot N_B}{M \cdot h_{\text{TOT}} \cdot \alpha} \times 100 \quad (1)$$

where B (mL) is the volume of base consumption, N_B (mol L⁻¹) the normality of the base, M (g) the mass of the protein, h_{TOT} (7.2 mequiv. g⁻¹ protein) the total number of peptide bonds in the substrate [10] and α the average degree of dissociation of α -amino groups in the β -lg–trypsin hydrolysis system. This last parameter was calculated according to Camacho et al. [11] and takes the value of 0.979 at pH 8.0.

Hydrolysis reactions were performed at six different initial protein concentrations (3, 9, 15, 23, 30 and 45 g L⁻¹), while the amount of enzyme added in each experiment was kept constant. The substrate/enzyme ratio (S/E) values used in the experiments were: 45, 131, 221, 344, 438 and 650 (w/w).

Samples of the incubation mixture were taken just before the addition of trypsin and during the hydrolysis reaction from 1 min up to 24 h at variable time intervals. The reaction was stopped by adding HCl and samples were stored at -20 °C prior to RP-HPLC analysis.

2.2. Peptide isolation and identification

A semi-preparative RP-HPLC system equipped with a fraction collector (Varian ProStar, Varian, USA) was used to isolate all the peptides present in the hydrolysate. The column used to perform the separation was a Dynamax Microsorb 300-8 C18 (250 × 21.4 mm, Varian, USA) preceded by a guard Dynamax Microsorb C18 column (Varian, USA). The 2 mL sample loop was used to inject the sample. A gradient of solvent A containing 0.1% trifluoroacetic acid (TFA) (v/v) in water and solvent B consisting of 0.1% TFA (v/v) in acetonitrile was used to achieve the elution of the peptides as follows: isocratic elution at 5% B for 8 min, linear gradient from 5% to 45% B in 142 min, linear gradient from 45% to 80% B in 20 min, linear gradient from 80% to 95% B in 5 min and return to starting conditions in 1 min. The flow rate was 10 mL min⁻¹, the column was used at ambient temperature and absorbance was measured at 214 nm. Total run time was 176 min. Ultrapure water (MilliQsystem, Millipore, USA), acetonitrile grade HPLC (Prolabo, Belgium) and TFA for UV analysis (Panreac, Spain) were used to prepare the eluents.

All the fractions obtained by the above procedure were first analyzed using a Voyager-DE STR matrix assisted laser desorption/ionization time-of-flight mass spectrometer (MALDI-TOF/MS) (Applied Biosystems, USA). Zip-Tip pipette tips containing C18 reverse-phase media (Millipore, USA) were used for sample preparation. The matrix used was α -cyano-4-hydroxy-cinnamic acid (10 mg/mL in 50% acetonitrile–0.1% TFA) (Sigma, USA). The mass range for MS analysis was between 500 and 3500 Da and MSCAL2 calibration kit (Sigma, USA) was used for external calibration. Mass spectrometry acquisition was performed in reflectron mode and positive polarity with an acceleration voltage of 25 kV. Spectra processing was carried out using Data Explorer (Applied Biosystems, USA), while peptide identification based on the molecular weight of the analyte was carried out using the FindPept tool on the ExPASy server (<http://web.expasy.org/findpept/>).

To ensure the correct identification of peptides, the fractions were also analyzed by direct-infusion electrospray mass spectrometry (ESI-MS/MS) in order to obtain their amino acid sequence. The equipment used was a Qstar XL (Applied Biosystems, USA), which is a quadrupole time-of-flight mass spectrometer equipped with an electrospray source. The MS/MS spectra obtained were analyzed using Analyst QS 1.1 (Applied Biosystems, USA).

2.3. Chromatographic analysis and peptide quantification

The separation of peptides and β -lg present in the reaction mixture was carried out by RP-HPLC. The analytical HPLC equipment consisted of an Agilent 1200 series chromatograph (Agilent Technologies, USA) equipped with a binary pump, auto sampler and a photodiode-array detector. The equipment was controlled by the Agilent ChemStation for Liquid Chromatography Systems software, which sets analysis parameters, data acquisition and data processing. Separations were performed on a Microsorb-MV C18 column (250 × 4.6 mm × 1/4 in., Varian, USA) at 25 °C and a flow rate of 1.0 mL min⁻¹. A gradient of the same solvents used in the semi-preparative RP-HPLC system was applied. Analytes were separated and eluted with the following programme: linear gradient from 5% to 45% B in 55 min, linear gradient from 45% to 90% B in 10 min, isocratic elution at 90% B for 10 min and then return to starting conditions in 2 min. Total analysis time per sample was 77 min. Elution was monitored at 214 nm to enable protein and peptide detection, and the sample injection volume was 20 μ L.

All samples were diluted with ultra-purified water and filtered through 0.45 μ m pore diameter cellulose acetate filters (Teknokroma, Spain). The fractions collected using the semi-preparative RP-HPLC system containing individual peptides were analyzed through the same analytical method in order to identify the peptides of the chromatographic profile by retention time.

The molar concentration of the peptides derived from β -lg was calculated from the peak area in the chromatograms according to Eq. (2) [12].

$$x_j = 1 \times 10^6 \left(\frac{A_j}{\varepsilon_j \cdot l \cdot v} \right) \cdot f \quad (2)$$

where x_j (μ M) is the concentration of the peptide j , A_j (AU min) the area of the HPLC peak of peptide j at 214 nm, l (0.6 cm) the path length of the UV cell, v (20 μ L) the injection volume, f (1000 μ L min⁻¹) the flow rate and ε_j (AUM⁻¹cm⁻¹) the molar extinction coefficient of peptide j at 214 nm calculated according to Kuipers and Gruppen [13].

2.4. Kinetics analysis and fitting procedure

For enzymatic hydrolysis in a batch system, the dynamic evolution of the substrate is described as:

$$\frac{dx}{dt} = -\rho_x \quad (3)$$

where x (mM) is the concentration of the intact protein and ρ_x (mM min⁻¹) is the reaction rate. The definition of the kinetic function ρ is the key element in the construction of the model. In general, the reaction rates of enzymatic proteolysis are assumed to follow either a Michaelis–Menten or a first-order kinetics, although it should be noted that competition for the active site of the enzyme between the original substrate and the intermediate peptides that are produced during hydrolysis could lead to the possibility of a product inhibition model. Common kinetic functions were evaluated for the degradation of the intact protein and, in the light of the experimental results for peptide concentration, a kinetic model for peptide release has been proposed. Least-squares fitting of the

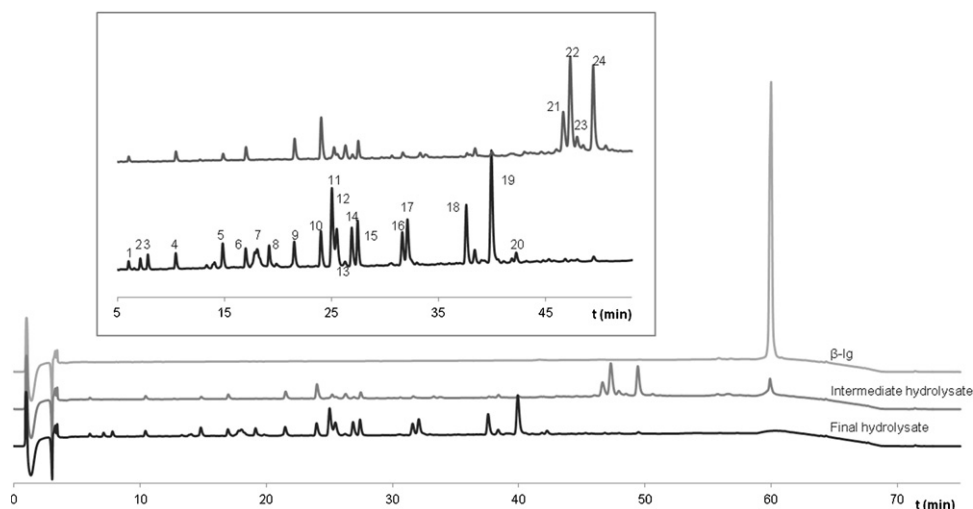


Fig. 1. Chromatographic profile of the intact protein, intermediate hydrolysate and final product of the hydrolysis.

experimental data was performed using the computer program Scientist for Windows Version 2.0. Statistical parameters, namely the coefficient of determination and the correlation between observed and calculated values are reported by the software as indicators of goodness-of-fit.

2.5. Statistical analysis

All experiments were performed in duplicate. The data obtained were reported as mean and standard deviation, and the difference between treatments was analyzed by one-way ANOVA using Microsoft Office Excel 2007 (Microsoft Co., USA). Significant differences were evaluated at $P < 0.05$. The data points plotted in the figures represent means $\pm$ standard deviations for the duplicate experiments. In the case of kinetic parameter estimations, the goodness-of-fit was evaluated by means of the coefficient of determination.

3. Results and discussion

3.1. Characterization of the hydrolysate: chromatographic profile and peptide identification

Typical chromatographic profiles of three samples taken at different DH are shown in Fig. 1. One of the profiles corresponds to the initial β -Ig solution and, as can be observed, the two main genetic variants of the protein (A and B) co-elute with a retention time of 59.9 min. The second profile shows an intermediate step in the course of the hydrolysis reaction (DH 5.44). The action of trypsin releases some peptides from the β -Ig, so the concentration of protein decreases while some new peaks appear. Finally, the third profile is of a sample taken at DH 9.82. At this point, the hydrolysis reaction is almost complete and comparison between these last two profiles shows that, while some peptides are released immediately when the reaction starts (peptides number 1 or 4), there are others (peptides number 18 or 19) whose formation depends on the release and subsequent degradation of intermediate products (peptides number 21–24). As can be seen in Fig. 1, good resolution of the chromatographic peaks during RP-HPLC allowed the monitoring of both β -Ig degradation and peptide formation on the basis of their peak areas.

Peak numbers on the chromatogram refer to peptide identification obtained by MALDI-TOF-MS and ESI-MS/MS, as reported in Table 1. Up to 18 peptide sequences were identified from the

fractions isolated by means of the semi-preparative HPLC system, allowing 85% coverage of the entire amino acid sequence. The only region which was not identified corresponds to β -Ig f(101–124). This fragment contains a free-cysteine residue that does not participate in disulfide linkage in the native protein. Consequently, the formation of aggregates during hydrolysis at basic pH is a possibility which may contribute to the difficulty in identifying this peptide. A similar observation was reported when using HPLC separation coupled with ion-trap mass analysis [14]. Furthermore, it was not possible to identify peptide number 2 mainly due to its low molecular weight. Likewise, although the molecular masses of peptides 20–24 were obtained, it was not possible to determine their amino acid sequences through the analytical techniques employed in this study. As can be observed in Fig. 1, these peptides are rapidly released in the first stage of the hydrolysis and are not present in the final sample; they are hence assumed to be intermediate products of the reaction. These peptides were not identified, or even analyzed, in previous studies by other authors [14,15] because only final hydrolysis samples are usually analyzed.

As can be seen in Table 1, MALDI-TOF-MS and ESI-MS/MS provide similar results. However, contrary to what occurs with MALDI-TOF-MS, ESI-MS/MS offers the possibility of sequencing peptides whose molecular mass is smaller than 500 Da, such as f(139–141) and f(136–138).

3.2. Hydrolysis of intact β -Ig

As stated in Section 1, the decrease in intact protein over time for all experiments was calculated from HPLC chromatograms using Eq. (2), while the DH was calculated according to the pH-stat technique via Eq. (1). Fig. 2 shows the time-course variation of β -Ig concentration and DH throughout the experiment in which the initial protein concentration was 45 g L^{-1} . As can be seen, although all the intact protein disappears in the first steps of the hydrolysis (the substrate concentration is zero after an hydrolysis time of 2 h), the DH continues to rise (from 6.48 at 2 h to 8.09 at 24 h). This implies that the hydrolysis reaction passes through some intermediate products before the final peptides are released. Thus, for example, peptides 21–24 in the chromatographic profile are rapidly formed at the beginning of the reaction, but are not present in the hydrolysate after 24 h (see Fig. 1).

Experimental data obtained for β -Ig concentration were used to estimate the parameters of a number of candidate kinetic models (data not shown). The best fitting of results was obtained when the

Table 1
Peptide identification through ESI-MS/MS and MALDI-TOF-MS analysis.

Peak number	m/z (MALDI-TOF-MS)	Peptide (FIndPept)	m/z (ESI-MS/MS) ^a	Peptide (MS/MS analysis)	Sequence ^c
1			331.23 (z: 1) ^b	ALK	f(139–141)
2					
3			409.20 (z: 1)	FDK	f(136–138)
4	573.45	IIAEK	573.34 (z: 1)	IIAEK	f(71–75)
5	916.46	IDALNENK	458.73 (z: 2)	IDALNENK	f(84–91)
6	673.51	GLDIQK	673.38 (z: 1)	GLDIQK	f(9–14)
7	2125.65 – 2183.65 – 2311.76	WEND(G)ECAQK + WEND(G)ECAQKK (Ag.)	427.24 (z: 2) – 709.30 (z: 3) – 728 (z: 3) – 771.34 (z: 3)		f(61–69) + f(61–70)
8	1245.6	TPEVDDEALEK	623.29 (z: 2)	TPEVDDEALEK	f(125–135)
9	933.56	LIVTQTMK	467.28 (z: 2)	LIVTQTMK	f(1–8)
10	837.5	ALPMHIR	419.24 (z: 2)	ALPMHIR	f(142–148)
11	696.37	VAGTWY	695.34 (z: 1)	VAGTWY	f(15–20)
12	674.43	IPAVFK	674.42 (z: 1)	IPAVFK	f(79–83)
13			452.29 (z: 2)	TKIPAVFK	f(76–83)
14	1065.56	VLVLDTDYK	533.29 (z: 2)	VLVLDTDYK	f(92–100)
15	1635.68	TPEVDDEALEKFDK	545.95 (z: 3)	TPEVDDEALEKFDK	f(125–138)
16	2906.99	WEND(G)ECAQKK + LSFNPTQLEEQCHI (Ag.)	969.45 (z: 3)	WEND(G)ECAQKK + LSFNPTQLEEQCHI	f(61–70) + f(149–162)
17	2720.25	WEND(G)ECAQK + LSFNPTQLEEQCHI (Ag.)	908.11 (z: 3)	WEND(G)ECAQK + LSFNPTQLEEQCHI	f(61–69) + f(149–162)
18	2030.13	SLAMASSDISLLDAQSAPLR	677.39 (z: 3)	SLAMASSDISLLDAQSAPLR	f(21–40)
19	2312.68	VYVEELKPTPEGDLLEILLQK	771.78 (z: 3)	VYVEELKPTPEGDLLEILLQK	f(41–60)
20			805.96 (z: 2) – 871.97 (z: 2)		
21			1003.92 (z: 5) – 1041.14 (z: 5) – 1254.64 (z: 4) – 1301.17 (z: 4)		
22					
23					
24			903.17 (z: 3)	VAGTWYSLAMASSDISLLDAQSAPLR	f(15–40)

^a MALDI-TOF-MS analysis produces monocharged analytes (z: 1).

^b The charges (z) of the analytes produced by ESI-MS/MS analysis are shown.

^c Amino acid interval within the sequence of the parent protein.

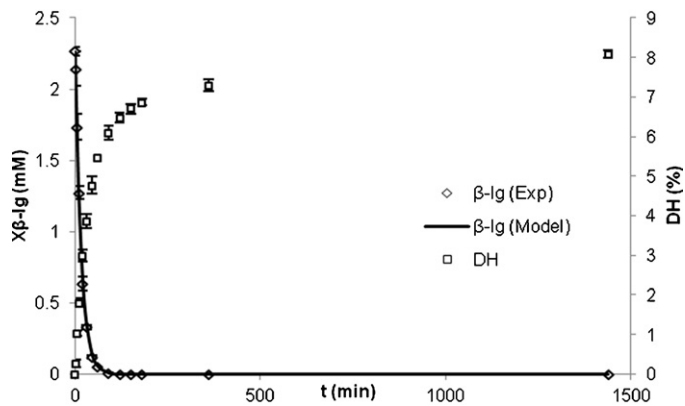


Fig. 2. Time-course variation of intact β -Ig concentration and DH throughout the course of the experiment. Initial β -Ig concentration: 45 g L^{-1} .

reaction rate follows a first-order kinetic equation (see Table 2). This means that the reaction rate can be expressed as:

$$\rho_x = k_a \cdot E \cdot x \quad (4)$$

Table 2
Degradation constant (k_a) estimations for first-order kinetics.

$X_{\beta\text{-Ig init}} (\text{g L}^{-1})$	k_a	R^2
45	0.0000657 ± 0.00000117	0.9996
30	0.000104 ± 0.00000251	0.9995
23	0.0000127 ± 0.00000303	0.9994
15	0.000157 ± 0.00000516	0.999
9	0.000187 ± 0.00000354	0.9998
3	0.000324 ± 0.0000206	0.998

where $k_a (\text{mL U}^{-1} \text{min}^{-1})$ is the degradation constant and $E (\text{U mL}^{-1})$ is the enzyme concentration. According to Table 2, the estimates of the first-order kinetic model have small standard deviations (in the worst case, this parameter is no more than 6.4% of the estimated k_a value) and the correlation factors are good enough to validate the degradation model.

Best fitting results obtained using a first-order kinetic equation for substrate degradation are not surprising. First-order kinetics is the consequence of a mechanism in which the rate-controlling step is the first attack on the tertiary structure of the protein. When working with β -lg, this hypothesis is supported by the globular conformation of the protein [16]. However, other authors have modeled enzymatic whey protein hydrolysis by means of product inhibition models [17] or by taking into account the deactivation of the enzyme [18], but in this case these models were not considered as the activity of the enzyme is in excess. The amount of trypsin added is high enough (it can hydrolyze $0.185 \text{ mol min}^{-1}$ of substrate) compared to the substrate concentration ($9 \mu\text{mol}$ in the most concentrated experiment) to be considered a constant value.

3.3. Time-dependent release of peptides: model development

The time-dependent release of the identified peptides is shown in Fig. 3. Fractions corresponding to peptide numbers 7, 16 and 17 (Fig. 3d) consist of peptide aggregates and the dynamics of the individual peptides are not discussed in this paper. As can be observed in Fig. 3, peptides exhibit different dynamic characteristics for any given experimental conditions and can be classified into three main groups. First of all, there are some peptides (Fig. 3a) whose formation is directly linked to the breakdown of intact β -lg; i.e., their dynamics will be best represented by first-order kinetics. Secondly, there is a group of peptides (Fig. 3b) which are formed rapidly in the

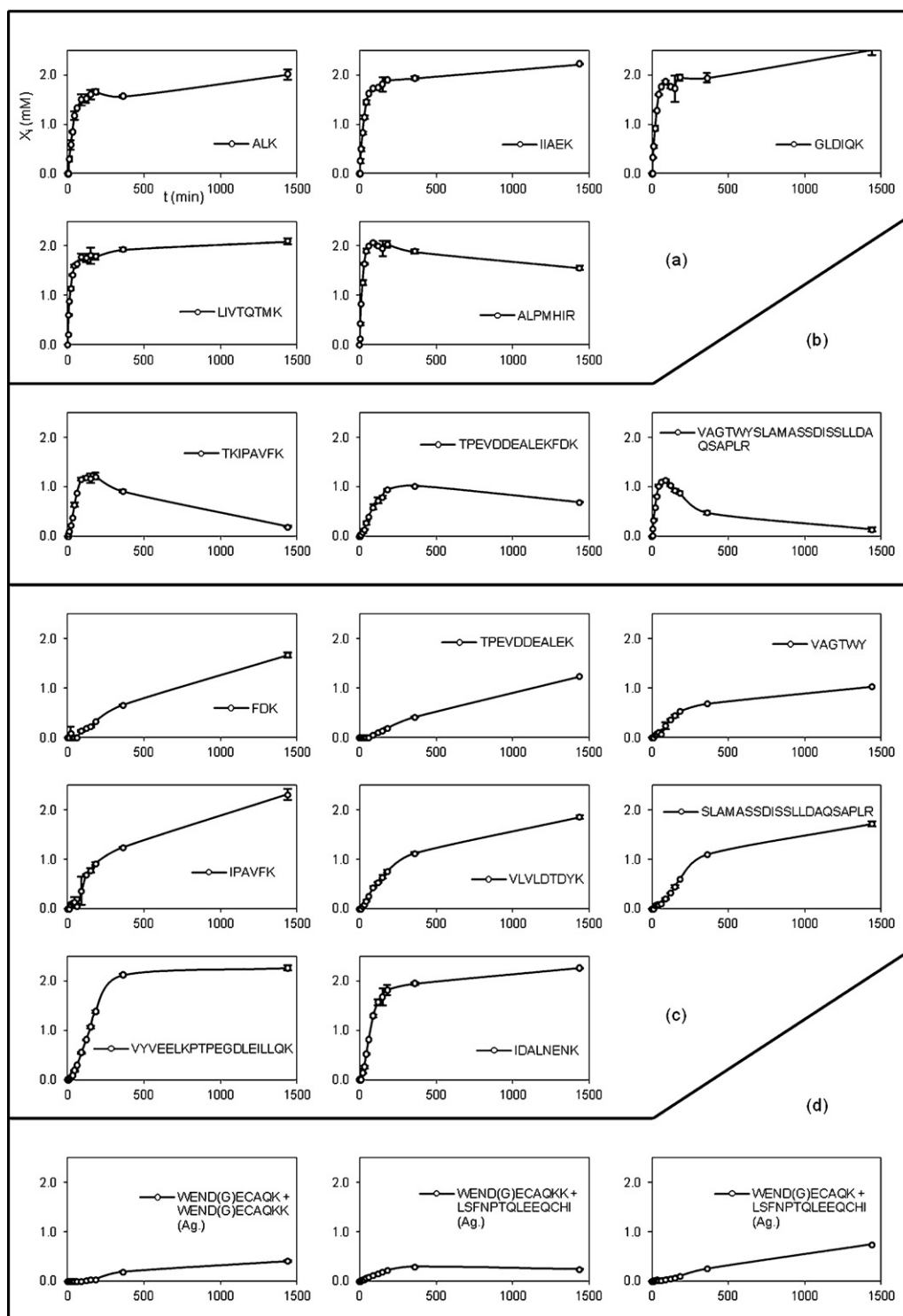


Fig. 3. Time-dependent release of identified peptides: (a) peptides directly derived from β -Ig, (b) intermediate products, (c) final peptides derived from intermediate products, and (d) peptides linked by disulfide bridges. Initial β -Ig concentration: 45 g L^{-1} .

first stage of the hydrolysis, but are then further hydrolyzed leading to the formation of smaller peptides. Finally, the release of some of the identified peptides (Fig. 3c) is affected by a delay time, probably due to the formation of intermediate products before their final release.

The observed differences in dynamic characteristics in Fig. 3 could be explained by the affinity of the enzyme for a particular scissile bond and its neighboring region (the group of amino acid residues around the peptide bond and their spatial conformation).

On the one hand, the tridimensional globular structure of the protein makes it difficult for the enzyme to access certain scissile bonds. Thus, peptides such as $f(1-8)$ and $f(142-148)$ are rapidly released because they occupy external positions within the protein (see Fig. 3a). On the other hand, the secondary specificity of the enzyme may play a role in the dynamics of peptide release. It is a well-known fact that trypsin has a narrow specificity and that it only hydrolyses peptidic bonds involving the carboxylic group of lysine and arginine residues. However, tryptic activity is

Table 3

Theoretical analysis of the physical accessibility (from very easy (+++), to difficult (–)) and secondary specificity of the enzyme (from favored (++) to inhibited (–)) and experimental $t_{1/2}$ values obtained for final peptides identified in the chromatographic profile.

Final product	Scissile bonds	Physical accessibility	Secondary specificity	$t_{1/2}$ (min)
f(1–8): LIVTQTMK	K ₈ –G ₉	+++	+	20
f(9–14): GLDIQK	K ₈ –G ₉ /K ₁₄ –V ₁₅	++	++	26
f(15–20): VAGTWY	K ₁₄ –V ₁₅ /Y ₂₀ –S ₂₁	+	Non specific	>1440
f(21–40): SLA...PLR	Y ₂₀ –S ₂₁ /R ₄₀ –V ₄₁	+	Non specific	420
f(41–60): VYV...LQK	R ₄₀ –V ₄₁ /K ₆₀ –W ₆₁	+	+	187
f(71–75): IIAEK	K ₇₀ –I ₇₁ /K ₇₅ –T ₇₆	+	++	30
f(79–83): IPAVFK	K ₇₇ –I ₇₈ /K ₈₃ –I ₈₄	+	–	301
f(84–91): IDALNENK	K ₈₃ –I ₈₄ /K ₉₁ –V ₉₂	0	0	80
f(92–100): VLVLDTDYK	K ₉₁ –V ₉₂ /K ₁₀₀ –K ₁₀₁	0	+	376
f(125–135): TPEVDDEALEK	R ₁₂₄ –I ₁₂₅ /K ₁₃₅ –F ₁₃₆	–	–	1308
f(136–138): FDK	K ₁₃₅ –F ₁₃₆ /K ₁₃₈ –A ₁₃₉	–	0	870
f(139–141): ALK	K ₁₃₈ –A ₁₃₉ /K ₁₄₁ –A ₁₄₂	0	++	43
f(142–148): ALPMHIR	K ₁₄₁ –A ₁₄₂ /R ₁₄₈ –L ₁₄₉	+	++	17

modulated by the five or six residues surrounding the cleavage site and these define its secondary specificity. Aliphatic residues, followed by aromatic and basic residues, are preferred in the positions extending toward the carboxy-terminal side of the peptides, whereas proline and acidic side chains are particularly disadvantageous. In addition, a carboxylic group in the second position toward the amino-terminal side of the amino acid chain decreases the rate of hydrolysis [19]. Thus, for example, although peptide f(139–141) is located on the outer surface of the protein, its rate of release is lower than expected (see Fig. 3a) due to the presence of an acidic residue in the second position toward the amino-terminal side of peptide bond K₁₃₈–A₁₃₉.

The $t_{1/2}$ values of the final peptides, defined as the hydrolysis time required for the appearance of 50% of the theoretical maximum amount of each peptide, were calculated. Table 3 shows $t_{1/2}$ values as well as the relation between these values, the secondary specificity of the enzyme and the physical accessibility to the peptide bonds that have to be cleaved in order to release each peptide. To evaluate this last parameter, the three-dimensional structure of β -lg was considered (see Fig. 4).

As can be appreciated, the lowest $t_{1/2}$ values (<60 min) correspond to peptides whose release is favored by both secondary specificity and physical accessibility. However, if one of these factors works counter to the action of trypsin, the $t_{1/2}$ values will increase. Note the high $t_{1/2}$ value obtained for peptides f(125–135) ($t_{1/2}$ = 1308 min) and f(136–138) ($t_{1/2}$ = 870 min). Both peptides are located within an α -helix motif (see Fig. 4), which hinders the physical approach of the enzyme, while the presence of proline and acidic residues surrounding the cleavage sites does not favor trypsin activity.

In view of the results shown in Fig. 3 and Table 3, both corresponding to the experiment in which the initial protein concentration was 45 g L^{–1}, a kinetic model has been proposed to explain peptide release. Fig. 5 shows its schematic representation. According to this kinetic approach, the action of trypsin on intact protein results in the release of some intermediate peptides, which act as new substrates, and some final products. As previously stated, the secondary specificity of the enzyme and local accessibility to a particular scissile bond are responsible for these results. As a general rule, the peptides directly derived from β -lg degradation are located in the N- and C-terminal areas of the protein, whereas the intermediate products corresponds to those regions which are less accessible to trypsin. However, peptide f(71–75) represents an exception to the above statements. Its quick release could be explained because of its external position in the three-dimensional structure of the protein and its amino acid composition, both which favor the action of trypsin. On the other hand, it seems that the amino acid sequence corresponding to f(76–138) constitutes a resistant core to trypsin action and the protection of scissile bonds

within this sequence could be justified by their neighboring amino acids or their poor physical accessibility (see Fig. 4 and Table 3). These observations are in accordance with the findings of Cheison et al. [15].

Based on the assumption of a first-order kinetic system, a set of differential equations was established in order to simulate the time-course release of the identified peptides according to the proposed model (see Appendix A).

As stated previously, the kinetic behavior of peptides corresponding to f(61–70), f(102–124) and f(149–162) is not discussed in this paper because they are present in the form of peptide aggregates and HPLC does not allow their proper monitoring as individual peptides.

Apparent constant rates (k_i) and accessibility constants (A_i) values were obtained from the comparison of experimental data and kinetic equations by means of the least-squares minimization procedure employed by Micromath Scientist. Table 4 shows the kinetic parameters as well as the coefficient of determination between

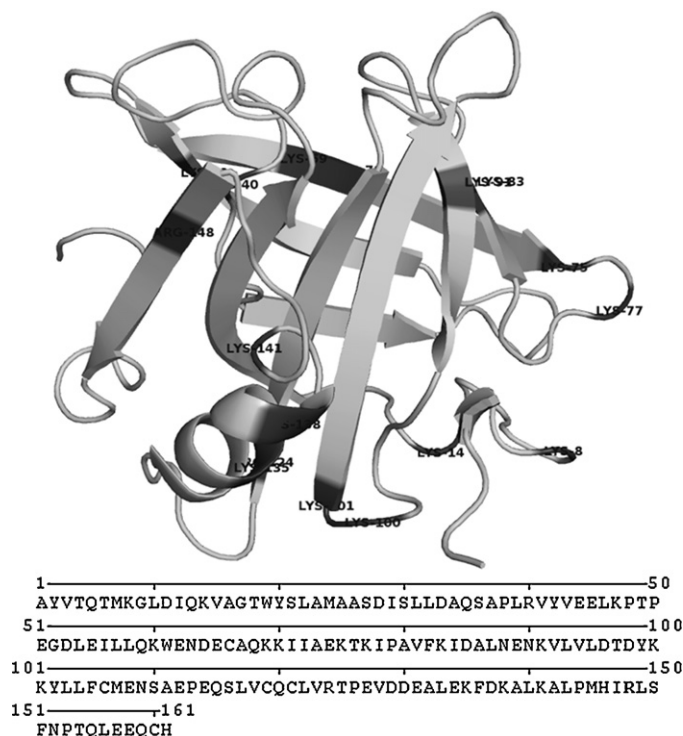


Fig. 4. Molecular structure of β -lg, with preferential cleavage sites of trypsin highlighted (compiled on <http://polyview.cchm.org> on July 10th, 2012).

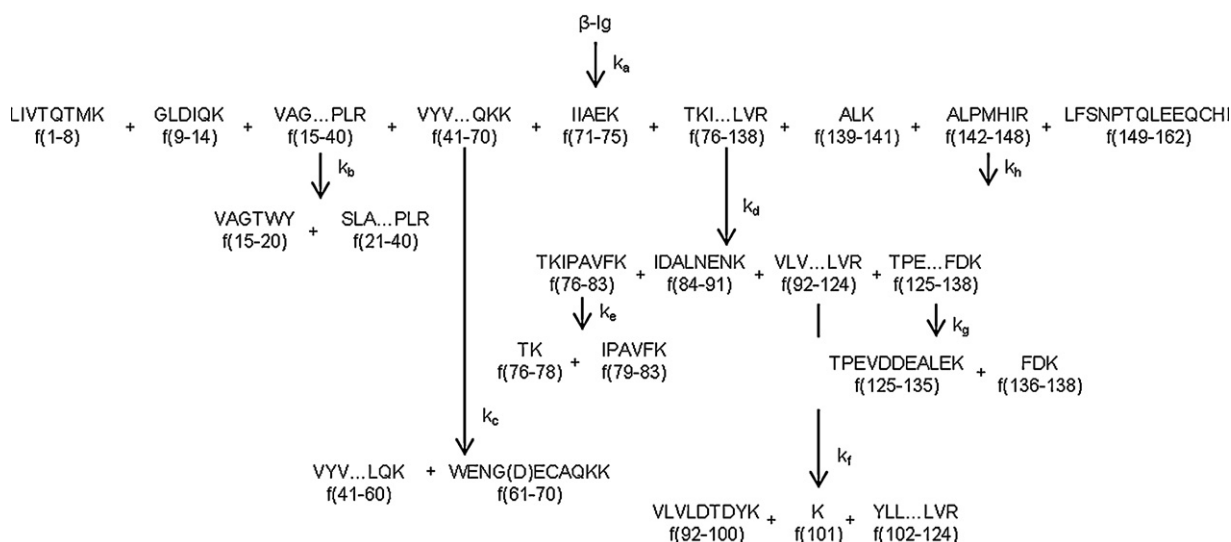


Fig. 5. Schematic representation of the proposed kinetic model.

Table 4
Kinetic parameters and coefficient of determination between experimental and simulated data obtained according to the proposed model. Initial β -Ig concentration: 45 g L⁻¹.

k_i (mL U ⁻¹ min ⁻¹)					
k_a	$6.57 \cdot 10^{-5} \pm 1.18 \cdot 10^{-6}$	k_d	$1.11 \cdot 10^{-5} \pm 4.00 \cdot 10^{-7}$	k_g	$1.83 \cdot 10^{-6} \pm 1.88 \cdot 10^{-7}$
k_b	$3.25 \cdot 10^{-6} \pm 2.61 \cdot 10^{-7}$	k_e	$4.81 \cdot 10^{-5} \pm 3.40 \cdot 10^{-7}$	k_h	$1.89 \cdot 10^{-7} \pm 2.81 \cdot 10^{-8}$
k_c	$5.33 \cdot 10^{-6} \pm 2.64 \cdot 10^{-7}$	k_f	$4.57 \cdot 10^{-6} \pm 5.09 \cdot 10^{-7}$		
Accessibility constants					
A_1	0.796 ± 0.030	A_3	0.565 ± 0.017	A_5	0.639 ± 0.030
A_2	0.805 ± 0.030	A_4	0.761 ± 0.030	A_6	0.889 ± 0.009
Coefficient of determination (R^2)					
$f(1-8)$	0.997	$f(71-75)$	0.98	$f(125-135)$	0.991
$f(9-14)$	0.989	$f(76-83)$	0.983	$f(136-138)$	0.948
$f(15-40)$	0.963	$f(78-83)$	0.962	$f(139-141)$	0.952
$f(15-20)$	0.964	$f(84-91)$	0.995	$f(142-148)$	0.998
$f(21-40)$	0.938	$f(92-100)$	0.93		
$f(41-60)$	0.98	$f(125-138)$	0.907		

experimental and simulated data obtained for the experiment in which the initial protein concentration was 45 g L⁻¹. The statistical data obtained seem to support the hypothesis that the hydrolysis mainly takes place via the proposed model.

As can be observed, k_h presents the minimum value among all the apparent constant rates. This is not unexpected, as peptide $f(142-148)$ should not be hydrolyzed by the action of trypsin. Its degradation phenomenon, only observed in the last stages of the reaction, might be assigned to the TPCK treatment of the trypsin that did not succeed in totally eliminating chymotrypsin activity [19]. More surprising is the rapid degradation of peptide $f(15-40)$. As in the case mentioned above, the digestion of this peptide might be due to chymotrypsin activity; in fact the amino acid tyrosine (Y) is one of the main substrates of this enzyme. However, k_b is an order of magnitude larger than k_h .

As regards the accessibility constants, their values are in agreement with the previously stated hypothesis. Thus, for example, the accessibility constant calculated to simulate the release of peptide $f(139-141)$ is 0.639, whereas it takes the value of 0.796 in the case of $f(1-8)$, as the neighboring amino acid of peptide $f(139-141)$ does not promote trypsin action and the scissile bond K_8-G_9 , the only one that needs to be cleaved in order to release peptide $f(1-8)$, is more physically accessible to the enzyme (see Fig. 4). The influence of accessibility constants in the prediction of peptides directly derived from β -Ig is shown in Fig. 6. As can be observed, if the simulation is performed solely using kinetic constant k_a , the

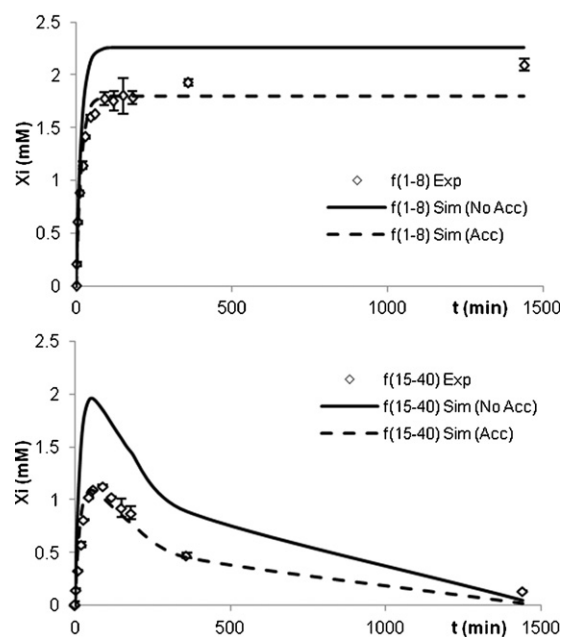


Fig. 6. Influence of the accessibility constant on the simulation of $f(1-8)$ and $f(15-40)$, peptides directly derived from β -Ig.

model overestimates experimental data. However, on introducing the accessibility constant, the fit between the proposed model and experimental data is much better.

4. Conclusions

The time-dependent release of peptides from tryptic digestion of β -lg at pH 8 and 37 °C was investigated in this paper. The identification of 18 peptides from β -lg, whose sequence coverage reached 85%, and the analytical methodology employed in this study allowed the quantification and monitoring of the released peptides. Although most of the identified peptides originated from specific trypsin action, the release of some peptides obtained through a chymotrypsin-like reaction was also observed.

Due to the globular conformation of the protein, the best fitting of results for β -lg degradation was obtained by means of a first-order kinetic, where the rate-controlling step is the attack on the tertiary structure of the protein. The fact that the DH continued to rise when all the intact protein had disappeared implies that the hydrolysis reaction passes through some intermediate products before the final peptides are released.

As regards peptide release, experimental data showed that their dynamics largely depends on the secondary specificity of the enzyme and the three-dimensional structure of the amino acid region surrounding a particular scissile bond. In this respect, the released peptides were classified into three different groups depending on their kinetics. Firstly, peptides mainly located in the N- and C-terminal areas of β -lg were directly released from the degradation of the intact protein. Secondly, the relative resistance to trypsin hydrolysis of the internal part of the protein indicated that the release of final products within this region implied the formation and subsequent degradation of intermediate peptides. As a consequence of this last observation, the release of the last group of peptides (final products located in the middle of the amino acid sequence of the protein) seemed to be affected by a delay time. The discrimination of tryptic susceptibility between different protein areas was compared in terms of the time-dependent release of peptides.

In view of experimental results, a kinetic model approach based on the assumption of first-order kinetics has been proposed. The main peculiarity of the model lies in the introduction of accessibility constants to simulate peptides directly derived from β -lg in order to correct the overestimation committed by not taking into account the secondary specificity and spatial conformation of the peptidic sequences involved in the process when optimizing the β -lg degradation constant value.

Acknowledgements

Author Ayoa Fernández acknowledges a Ph.D. fellowship from the Severo Ochoa programme (Principado de Asturias Government).

Appendix A. Kinetic model equations

Based on the assumption of a first-order kinetic system, a set of differential equations was established in order to simulate the time-course release of the identified peptides according to the proposed model

$$\frac{dx_{\beta\text{-lg}}}{dt} = -k_a \cdot x_{\beta\text{-lg}} \cdot E \quad (\text{A.1})$$

$$\frac{dx_{f(1-8)}}{dt} = k_a \cdot x_{\beta\text{-lg}} \cdot E \cdot A_1 \quad (\text{A.2})$$

$$\frac{dx_{f(9-14)}}{dt} = k_a \cdot x_{\beta\text{-lg}} \cdot E \cdot A_2 \quad (\text{A.3})$$

$$\frac{dx_{f(15-40)}}{dt} = k_a \cdot x_{\beta\text{-lg}} \cdot E \cdot A_3 - k_b \cdot x_{f(15-40)} \cdot E \quad (\text{A.4})$$

$$\frac{dx_{f(15-20)}}{dt} = k_b \cdot x_{f(15-40)} \cdot E \quad (\text{A.5a})$$

$$\frac{dx_{f(21-40)}}{dt} = k_b \cdot x_{f(15-40)} \cdot E \quad (\text{A.5b})$$

$$\frac{dx_{f(41-60)}}{dt} = k_c \cdot x_{f(41-70)} \cdot E \quad (\text{A.6})$$

$$\frac{dx_{f(71-75)}}{dt} = k_a \cdot x_{\beta\text{-lg}} \cdot E \cdot A_4 \quad (\text{A.7})$$

$$\frac{dx_{f(76-83)}}{dt} = k_d \cdot x_{f(76-138)} \cdot E - k_e \cdot x_{f(76-83)} \cdot E \quad (\text{A.8})$$

$$\frac{dx_{f(78-83)}}{dt} = k_e \cdot x_{f(76-83)} \cdot E \quad (\text{A.9})$$

$$\frac{dx_{f(84-91)}}{dt} = k_d \cdot x_{f(76-138)} \cdot E \quad (\text{A.10})$$

$$\frac{dx_{f(92-100)}}{dt} = k_f \cdot x_{f(92-124)} \cdot E \quad (\text{A.11})$$

$$\frac{dx_{f(125-138)}}{dt} = k_d \cdot x_{f(76-138)} \cdot E - k_g \cdot x_{f(125-138)} \cdot E \quad (\text{A.12})$$

$$\frac{dx_{f(125-135)}}{dt} = k_g \cdot x_{f(125-138)} \cdot E \quad (\text{A.13})$$

$$\frac{dx_{f(136-138)}}{dt} = k_g \cdot x_{f(125-138)} \cdot E \quad (\text{A.14})$$

$$\frac{dx_{f(139-141)}}{dt} = k_a \cdot x_{\beta\text{-lg}} \cdot E \cdot A_5 \quad (\text{A.15})$$

$$\frac{dx_{f(142-148)}}{dt} = k_a \cdot x_{\beta\text{-lg}} \cdot E \cdot A_6 - k_h \cdot x_{f(142-148)} \cdot E \quad (\text{A.16})$$

where k_i ($\text{mLU}^{-1} \text{min}^{-1}$) are the apparent constant rates, E (U mL^{-1}) is the enzyme concentration and A_i are the accessibility constants employed in the simulation of the peptides directly derived from β -lg in order to correct the overestimation committed by not taking into account the secondary specificity and spatial conformation of the peptidic sequence when optimizing the k_a value. It should be noted that the k_a value is obtained directly from β -lg degradation. The accessibility constants take values ranging from zero to one. The higher their value, the better the combination between secondary specificity of the enzyme and spatial conformation of the amino acid sequence surrounding the scissile bond.

As it was not possible to calculate the concentration of peptides $f(41-70)$ or $f(76-138)$ through the HPLC profile, the following mass balances were considered:

$$x_{f(41-70)} = x_{\beta\text{-lg}_0} - x_{f(41-60)} \quad (\text{A.17})$$

$$x_{f(76-138)} = x_{\beta\text{-lg}_0} - x_{f(84-91)} \quad (\text{A.18})$$

$$x_{f(92-124)} = x_{\beta\text{-lg}_0} - x_{f(76-138)} - x_{f(92-100)} \quad (\text{A.19})$$

References

- [1] D.D. Kitts, K. Weiler, Bioactive proteins and peptides from food sources. Application of bioprocesses used in isolation and recovery, *Curr. Pharm. Des.* 9 (2003) 1309–1323.
- [2] F.A. Riera, A. Fernández, C. Muro, Cheese whey as a source of active peptides: production, analysis, purification and animal and human trials, in: N. Gotsiridze-Columbus (Ed.), *Whey: Types, Composition and Health Implications*, Nova Science Publishers, New York, 2012.
- [3] R. Jost, J.S. Monti, J. Pahud, Whey protein allergenicity and its reduction by technological means, *Food Technol.* 41 (1987) 118–121.

- [4] E.A. Foegeding, J.P. Davis, D. Doucet, K. McGuffey, Advances in modifying and understanding whey protein functionality, *Trends Food Sci. Tech.* 13 (2002) 151–159.
- [5] J. Otte, Z.Y. Ju, M. Faergemand, S.B. Lomholt, K.B. Qvist, Protease-induced aggregation and gelation of whey proteins, *J. Food Sci.* 61 (1996) 911–915.
- [6] R. Muñoz-Tamayo, J. de Groot, P.A. Wierenga, H. Gruppen, M.H. Zwietering, L. Sijtsma, Modeling peptide formation during the hydrolysis of β -casein by *Lactococcus lactis*, *Process Biochem.* 47 (2012) 83–93.
- [7] K. Olsen, J. Otte, L.H. Skibsted, Steady-state kinetics and thermodynamics of the hydrolysis of β -lactoglobulin by trypsin, *J. Agric. Food Chem.* 48 (2000) 3086–3089.
- [8] M.V.T. Mota, I.M.P.L.V.O. Ferreira, M.B.P. Oliveira, C. Rocha, J.A. Teixeira, D. Torres, M.P. Gonçalves, Trypsin hydrolysis of whey protein concentrates: characterization using multivariate data analysis, *Food Chem.* 94 (2006) 278–286.
- [9] J. Adler-Nissen, *Enzymatic hydrolysis of food proteins*, Elsevier Applied Science Publishers, London, 1986.
- [10] S.C. Cheison, M. Schmitt, E. Leeb, T. Letzel, U. Kulozik, Influence of temperature and degree of hydrolysis on the peptide composition of trypsin hydrolysates of β -lactoglobulin: analysis by LC–ESI-TOF/MS, *Food Chem.* 121 (2010) 457–467.
- [11] F. Camacho, P. González-Tello, M.P. Páez-Dueñas, E.M. Guadix, A. Guadix, Correlation of base consumption with the degree of hydrolysis in enzymatic protein hydrolysis, *J. Dairy Res.* 68 (2001) 251–265.
- [12] R. Muñoz-Tamayo, J. de Groot, E. Bakx, P.A. Wierenga, H. Gruppen, M.H. Zwietering, L. Sijtsma, Hydrolysis of β -casein by the cell-envelope-located PI-type protease of *Lactococcus lactis*: a modeling approach, *Int. Dairy J.* 21 (2011) 755–762.
- [13] J. Bas, H. Kuipers, H. Gruppen, Prediction of molar extinction coefficients of proteins and peptides using UV absorption of the constituent amino acids at 214 nm to enable quantitative reverse phase high-performance liquid chromatography–mass spectrometry analysis, *J. Agr. Food Chem.* 55 (2007) 5445–5451.
- [14] J.F. Lapointe, S.F. Gauthier, Y. Pouliot, C. Bouchard, Characterization of interactions between β -lactoglobulin tryptic peptides and a nanofiltration membrane: impact on the surface membrane properties as determined by contact angle measurements, *J. Membr. Sci.* 261 (2005) 36–48.
- [15] S.C. Cheison, E. Leeb, T. Letzel, U. Kulozik, Influence of buffer type and concentration on the peptide composition of trypsin hydrolysates of β -lactoglobulin, *Food Chem.* 125 (2011) 121–127.
- [16] M.M. Vorob'ev, I. Levicheva, V.M. Belikov, Initial kinetics of milk protein hydrolysis by chymotrypsin, *Prikl. Biokhim. Mikrobiol.* 32 (1996) 237–241.
- [17] R. Sousa, G.P. Lopes, P.W. Tardioli, R.L.C. Giordano, P.I.F. Almeida, R.C. Giordano, Kinetic model for whey protein hydrolysis by alcalase multicomponent-immobilized on agarose gel particles, *Braz. J. Chem. Eng.* 21 (2004) 147–153.
- [18] Q. Wei, H. Zhimin, Enzymatic hydrolysis of protein: mechanism and kinetic model, *Front. Chem. China* 3 (2006) 308–314.
- [19] J. Tauzin, L. Miclo, S. Roth, D. Mollé, J.L. Gaillard, Tryptic hydrolysis of bovine α S2-casein: identification and release kinetics of peptides, *Int. Dairy J.* 13 (2003) 15–27.