Monitoring the large scale production of antihypertensive peptides RYLGY and AYFYPEL by HPLC-MS

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Abstract

This work reports the quantitative analysis of two novel antihypertensive peptides α_{s1}-CN f(90-94), with sequence RYLGY, and α_{s1}-CN f(143-149), with sequence AYFYPEL, by high-performance liquid chromatography–mass spectrometry in food grade hydrolysates of milk proteins. The method was validated and showed sufficient specificity, reproducibility, linearity and recovery. Linear calibrations of the molecular ions *m/z* 671.2 and 902.3 were selected for the determination of the peptides RYLGY and AYFYPEL, respectively, and showed good statistical results ($R^2 \geq 0.995$ and with no significant lack-of-fit). The simplicity of RP-HPLC-MS method allowed the automated quantification of both antihypertensive peptides without any sample pretreatment. The application of this method permitted the evaluation of some hydrolysis variables, i.e. substrate, temperature, hydrolysis time or enzyme/substrate ratio, on the formation of antihypertensive peptides. The quantitative analysis of RYLGY and AYFYPEL showed that ultrafiltration was not effective to improve the content in active peptides, containing the hydrolysates and their respective permeates similar peptide amounts.

Keywords Antihypertensive peptides, milk protein hydrolysate, mass spectrometry, functional ingredient, quantitative analysis.

Introduction

Food-derived peptides can exert different physiological effects in the organism, including antimicrobial properties, antihypertensive activities, cholesterol-lowering effects, antithrombotic and antioxidant actions, enhancement of mineral absorption, cyto- or immunomodulatory abilities, and/or opioid activity [1]. Among these, antihypertensive peptides are of special interest due to the prevalence of hypertension in World's population [2]. In borderline hypertensive subjects, they may represent a healthier and natural alternative to common drugs, like captopril, which may cause several side effects, such as hypotension, reduced renal function, cough, and fetal abnormalities [3].

Various food derived antihypertensive peptides have been described and extensively reviewed so far (for example, see [4-5]). However, antihypertensive peptides are only minor constituents in highly complex food matrixes, and thus, their identification, characterization, and quantification is a challenging task in food technology. The quantitative determination of antihypertensive peptides is indispensable to determine their minimal dose to show antihypertensive effects. Further, it is essential to continuously monitor the amount of a specific peptide produced during a hydrolytic or fermentative industrial process, and to asses the adequate content in the final product with the beneficial effect of blood pressure reduction [6, 7].

There are several products currently commercialized with antihypertensive claims, such as, Calpis and Evolus®. These products contain peptides VPP and/or IPP as bioactive tripeptides. Most interest has been focused on the demonstration of the antihypertensive effects *in vitro,* in model animals and in humans [8-10] (for a recent review, see [11]). In clinical trials, different doses of VPP and IPP have been used to achieve antihypertensive effects, between 2.6 and 5.6 mg at day [12]. In addition, some studies suggested a dose-dependent antihypertensive effect [13, 14]. Due to the relevance of the final daily intake of the tripeptides, robust and simple quantitative methods have to be developed to ensure the amount of these peptides in the final product [15-18].

In this context, mass spectrometry (MS) is an essential tool for identification, characterization, and quantification of peptides (for recent reviews, see [19, 20]). In the last years, different MS strategies have been applied to develop more accurate and specific quantitative methods; most of them combine the high resolving power of high performance liquid chromatography with the excellent selectivity and sensitivity of mass spectrometric detection [21]. In fact, RP-HPLC-MS, MS/MS and MS^3 methods have been successfully developed for the determination of antihypertensive peptides in different food matrixes, i.e. hydrolysates [18, 22], fermented milk [23] and cheeses [15, 16], but also in biological samples [24]. Peptide quantification generally requires an efficient sample pretreatment in order to remove interfering compounds thereby avoiding

instrument pollution or ion suppression, i.e. deproteinization by precipitation, solid- and liquid-phase extraction [18, 22, 25]. However, pretreatment results in more tedious analysis and poor recoveries, besides clean-up methods like solid-phase extraction could affect to the determination of hydrophilic peptides [24].

Casein is an excellent substrate to produce peptides with an antihypertensive effect [26-28]. Recently, Contreras et al. [29] have characterized two novel antihypertensive peptides derived from a peptic hydrolysate of casein, the peptides α_{s1}-casein f(90-94) and α_{s1}-casein f(143-149), corresponded to the sequences RYLGY and AYFYPEL, respectively. Both peptides showed angiotensin converting enzyme (ACE)-inhibitory activity with IC_{50} values as low as 0.71 μM and 6.58 μM, respectively, and also demonstrated significant antihypertensive activity when they were orally administered to spontaneously hypertensive rats at 5 mg/kg of body weight. The antihypertensive activity of each of them was similar to that found for VPP orally administered at the same dose. It is planned to perform some human intervention studies with milk protein hydrolysates containing these two peptides, RYLGY and AYFYPEL, and therefore, the exact administered dose has to be accurately determined.

The aim of the present work was to develop a robust and rapid analytical method with minimal sample treatment for the simultaneous quantification of the peptides RYLGY and AYFYPEL by RP-HPLC-MS, and MS/MS analysis. This method could be used for the routine determination of these peptides in products based on milk protein hydrolysates.

Materials and methods

Production of milk protein food grade hydrolysates

Two different products based on milk proteins, referred as powdered milk product A and powdered milk product B, were employed to prepare hydrolysates at laboratory scale or to produce hydrolysates at pilot scale by Innaves, S.A. (Porriño, Pontevedra, Spain). Powdered milk products A and B were resuspended in water at concentrations ranged between 6-30% (w/v). To prepare some hydrolysates powdered milk product A was enriched with commercial casein (Promilk 85, Arras Cedex, France) at 3 % (w/v). The suspensions were adjusted to pH 2.3-3.3 with 1 M food grade HCl (food grade, Aditio, Panreac Quimica, S.A.U., Castellar del Vallès, Spain) and digested with food grade pepsin (porcine gastric mucosa, 1:3000, Biocatalysts, Cardiff, UK) at different ratios. The hydrolysis was carried out between 37 and 50ºC with shaking at 150 rpm.

Pepsin was added at the beginning of the hydrolysis process and a second addition was made after several hours of hydrolysis, depending on the final hydrolysis time. Aliquots of the hydrolysates were taken at 4, 6, 8, 10 and/or 24 h. Hydrolysis was stopped by heating (> 80ºC) for 15 min and the pH was adjusted to 4.0 or

6.5 by addition of 1M KOH (food grade, Aditio, Panreac Quimica, S.A.U.). The hydrolysates were centrifuged at 16,000 × *g* for 15 min at 5ºC and the supernatants collected and lyophilized. Samples were kept at -20ºC until analysis.

The protein concentration of the supernatants after hydrolysis was determined by Kjeldahl method [30]. Previous to MS analysis, liquid samples were diluted 1/12 in Milli-Q water (Millipore, Bedford, MA, USA), whereas the freeze-dried samples were prepared at 5 mg/mL.

Preparation of standard solutions

The peptides RYLGY and AYFYPEL, synthesized by Fmoc solid-phase synthesis, were purchased from GenScript Corp. (Piscataway, NJ, USA). Their purity was higher than 95%. Stock solutions of 1 mg/mL of the synthetic peptides were prepared and mixed in Milli-Q deionized water (Millipore). Seven calibration points were obtained from 1 to 80 µg/mL.

Analysis by RP-HPLC-MS and MS/MS

RP-HPLC separations of the hydrolysates were performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 ion trap (Bruker Daltonik GmbH, Bremen, Germany) and equipped with an electrospray ionization source, as previously described [23]. The variable-wavelength detector was set at 214 nm. A C_{18}-guard column (Nova-Pak® 20 mm x 3.9 x 4 µm of particle size; Waters Corp., Milford, MA, USA) was used to protect the analytical column (HiPore® RP318 C_{18} column 250 x 4.6 mm and 5 µm of particle size; Bio-Rad, Richmond, CA, USA). The samples were eluted at 0.8 mL/min with a linear gradient from 0 to 45 % of solvent B (acetonitrile and TFA, 1000:0.270, v/v) in solvent A (water and TFA, 1000:0.370, v/v) in 60 min. The injection volume was 50 µL and duplicate of injection was made for each point of the standard curve and the samples. The flow from HPLC was divided approximately 1:3 previous to ionization source, and the first 6 min of the eluent flow was directed to waste to reduce salt deposit on the transfer capillary of the MS instrument and to reduce interferences. For HPLC-MS, spectra were recorded over the mass-to-charge (*m/z*) range of 100 to 1500. For quantitative purposes, tandem mass spectrometry was applied by using a signal threshold of 10,000, a window width of 4.0 *m/z*, and a voltage ramp going from 0.3 to 2 V. Helium was used as collision gas with an estimated pressure of 5×10^{-3} bar. About 15 spectra were averaged in the MS analyses and about 5 spectra in the tandem MS analyses. Using Data AnalysisTM (version 3.0; Bruker

Daltoniks), the *m/z* spectral data were processed and transformed to spectra representing mass values. The acquired MS/MS spectra were interpreted using BioTools (version 2.1; Bruker Daltoniks).

The quantification for RP-HPLC-MS and MS/MS was performed by representing the peak area obtained by MS and MS/MS analysis vs. the peptide concentration. In RP-HPLC-MS analysis, plots were made with the peak area of the molecular ions with *m/z* value of 671.2 and 902.3, corresponding to RYLGY and AYFYPEL, and their sodium and potassium adducts (*m/z* 693.2, 709.2 and 924.3, 940.3, respectively). The determination by RP-HPLC-MS/MS analysis was based on the peak of area of the most abundant product ions derived from RYLGY, with *m/z* value of 274.9, 508.1 and 654.2, and from AYFYPEL, with *m/z* value of 357.9, 521.2, 545.1 and 771.2.

Measurement of ACE-inhibitory activity

ACE inhibitory activity was measured by fluorescence using the method of Sentrandreu and Toldrá [31] and modified by Quirós et al. [32]. The ACE inhibitory activity of the samples was expressed as IC_{50} (protein concentration required to inhibit the original ACE activity by 50%) and was determined by triplicate.

Statistical method

Linear and polynomial regression for the calibration curves and non-linear regression for estimation of IC_{50} were calculated with Statgraphics Centurion XV version 15.2 (StatPoint Technologies, Inc., Warrenton, Virginia, USA, www.statgraphics.com) and GraphPad Prism version 4.03 (GraphPad Software Inc., San Diego, CA, USA, www.graphpad.com).

Results and discussion

Selection of analysis conditions

In the present work, MS/MS analysis have been evaluated for the rapid and simultaneous quantification of the antihypertensive peptides RYLGY and AYFYPEL in food grade hydrolysates with minimal sample treatment. The RP-HPLC-MS analysis of the hydrolysates revealed a complex mixture of peptides. To illustrate the complexity, Fig. 1a shows the total ion current of a powdered milk product A hydrolysed at pilot scale. One of the peptides of interest, RYLGY eluted between 31.1 and 31.7 min and the MS analysis revealed that RYLGY

(*m/z* 671.2) was resolved as a single chromatographic peak, where ions corresponding to its sodium and potassium adducts (*m/z* 693.2 and 709.2) were also observed. However, in other hydrolysates, RYLGY was not efficiently separated with the employed solvent gradient. Peptide AYFYPEL (*m/z* 902.3) co-eluted, between 45.3 and 45.9 min, together with other peptides, namely, ions with *m/z* values of 994.9, 1009.0 and 1039.3. Additionally to sodium and potassium adducts of AYFYPEL (*m/z* 924.3 and 940.3, respectively) that showed high intensity, some fragment ions derived from the break down of the AYFYPEL backbone, i.e. *m/z* 357.9 (y_3 ion), 545.1 (b_4 ion) and 668.3 (y_5 ion), were also observed in MS analysis. By extracting the molecular ion of RYLGY, a single peak was observed (Fig. 1b), being more specific than the joint extraction of ions *m/z* 671.2, 693.2, 709.2. It was due to the presence of the doubly charged ion *m/z* 693.2 in the hydrolysate. An adequate chromatographic method is required to selectively separate AYFYPEL (*m/z* 902.3) from other isobaric peptides generated during milk protein hydrolysis. The extracted ion chromatogram of the ion *m/z* 902.3 is shown in Fig. 1c. The extraction of the ion *m/z* 902.3 plus its sodium and potassium adducts (*m/z* 924.3 and 940.3, respectively) improve the MS signal. However, in this case, ions with *m/z* values of 940.3 and 938.2 and monoisotopic mass of 940.3 and 1875.8, respectively, were observed, but separated from AYFYPEL. Although the selectivity and intensity of the MS signal were adequate to perform a quantitative determination of both peptides, the response obtained by MS/MS analysis was also evaluated.

The MS/MS spectrum of the peptide RYLGY (Fig. 2a) revealed a series of N-terminal fragments, mainly *b*-type ions, probably due to the presence of an arginine residue in the N-terminal position [33, 34]. However, the most prominent fragment of the spectrum was the ion $[M+H]^+$-NH_3. The MS/MS spectrum of AYFYPEL (Fig. 2b) revealed various major fragments, i.e. ions with *m/z* values of 357.9 (y_3), 521.2 (y_4), 545.1 (b_4) and 771.2 (b_6), where the relatively high ratio of the y_3 and b_4 ions may be related with the presence of a proline residue [35]. Finally, the extraction of the main product ions derived from RYLGY (*m/z* 274.9, 508.1 and 654.2) resulted in an unique peak, being more specific than the extraction of AYFYPEL fragments (*m/z* 357.9, 521.2, 545.2 and 771.2), where various peaks were observed. In fact, protein hydrolysis with broad specificity enzymes, such as pepsin, releases a large number of peptides, some of them sharing similar sequences. These peptides may generate some identical fragments by MS/MS analysis. For example, the fragment ion *m/z* 357.9 (y_3) derived from AYFYPEL (Fig. 2b), was also generated by tandem MS of the peptide identified as YFYPEL, with a retention time between 44.6 and 45.2 min.

Method validation

Linear and polynomial regression models were applied for the calibration curves of RYLGY and AYFYPEL obtained by the analysis of the peak area vs. concentration of the standard peptides dissolved in Milli-Q water by

using two replicates at the range of concentration indicated in Table 1 and analysis by HPLC-MS and MS/MS. In the MS dimension, two different curves were obtained taking into account the molecular ion of each peptide or the molecular ion and its corresponding sodium and potassium adducts. In the MS/MS dimension, one or various fragments were selected to calculate the calibration curves. Regression parameters and statistical properties are shown in Table 1. All models showed a P-value for lack-of-fit test > 0.05. Fits showed good linearity in the concentration range from 1 to 20 μg/mL for RYLGY and 1 to 40 μg/mL for AYFYPEL by MS analysis. By MS/MS analysis, the range of linearity for RYLGY fragments was between 1 to 20 μg/mL, whereas for AYFYPEL was between 4 to 80 μg/mL. A second-degree polynomial equation was applied to describe the calibration curves when the standard concentration was incremented (from 1 to 80 μg/mL) because the MS response did not exhibit sufficient linear behavior. The values for the coefficient of determination, expressed as R^2, were generally higher than 0.99, and indicated that fits were acceptable but, in general, fits were better for curves obtained by MS analysis than by MS/MS.

Finally, linear calibrations of RYLGY and AYFYPEL using their respectively molecular ions with *m/z* 671.2 and 902.3 were selected for further analysis. The R^2 values were 0.999 for RYLGY and 0.995 for AYFYPEL. In this case, the standard deviation of residuals, expressed as a percentage of the mean value of the response (CV), was better than by using the molecular ions together with sodium and potassium adducts or fragment ions (Table 1).

Recovery was calculated as (the amount found in the spiked sample − the amount found in the sample) × 100/the amount added. The mean value for each peptide was determined from the recovery experiments by using two different concentrations of the synthetic peptides (10 and 20 μg/mL) added to a pilot scale hydrolysate, and triplicate injections of each point were performed. The mean recovery values were 119.5% and 114.5% by HPLC–MS analysis of the molecular ion of RYLGY (*m/z* 671.2) and AYFYPEL (*m/z* 902.3), and 88.6% and 96.7% by the analysis of the molecular ion together with sodium and potassium adducts of RYLGY (*m/z* 671.2, 693.2, 709.2) and AYFYPEL (*m/z* 902.3, 924.3, 940.3). Repeatability was estimated by the relative standard deviation (RSD) of the peak areas for six consecutive analysis of a hydrolysate at pilot scale. The values of RSD obtained by the HPLC-MS analysis of the molecular ions *m/z* 671.2 and 902.3 were 2.1 % and 5.6 %, respectively, and by the corresponding analysis of the molecular ions together with sodium and potassium adducts were 1.7% and 4.4%, respectively. Moreover, to determine reproducibility, 10 consecutive analyses of the hydrolysate were performed on two different days. This resulted in relative standard deviations of the areas of 8.4% and 9.5% by the analysis of the molecular ions *m/z* 671.2 and 902.3, and 4.2% and 7.5% by the analysis of the molecular ions and their corresponding adducts, respectively. Overall, these results demonstrate an

232 acceptable level of recovery, repeatability and reproducibility in comparison with other previously reported
233 methods, HPLC-UV [17,18] and HPLC-MS^n [15, 22, 23, 24].
234
235 The calibration curve obtained by HPLC-MS, using the peak area of ions *m/z* 671.2 and 902.3, was used
236 for quantification. The detection and quantification limits were estimated as the peptide concentration giving a
237 signal equal to the blank signal plus 3 and 10 standard deviations of the blanks, respectively [36]. The values
238 obtained were 0.002 and 0.005 μg/mL for the detection limit and 0.004 and 0.013 μg/mL for the quantification
239 limit of RYLGY and AYFYPEL, respectively. These levels are similar to that found for the ACE-inhibitory di-
240 peptides AF, YP and WY determined by an innovative HPLC method combined with replicate heart-cut column-
241 switching technique [37], and lower than for peptides LHLPLP determined by HPLC-MS/MS [23], VPP and IPP
242 by HPLC-UV [17]. These detection limits are sufficient for the purpose of the method since it is intended to
243 quantify amounts higher than 2.5 μg/mL, i.e., 0.5 mg/g of dried product.
244
245 Quantification of the peptides RYLGY and AYFYPEL in food grade hydrolysates
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247 *Effects of time, enzyme/substrate ratio and temperature*
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249 Before production of milk protein hydrolysates at pilot scale, various food grade hydrolysis were carried out at
250 laboratory scale, to evaluate some factors like, time, enzyme/substrate ratio, and temperature. Firstly, powdered
251 milk product A (10 %, w/v) was hydrolyzed at 37ºC with two different enzyme/substrate ratios (low and high
252 ratio) and at different hydrolysis times (4, 6, 8, 10 and 24 h) (Fig. 3). The content of RYLGY increased with the
253 hydrolysis time and the enzyme/substrate ratio. However, these factors caused an opposite effect upon
254 AYFYPEL content. Furthermore, when powdered milk product A was hydrolyzed during 24 h, RYLGY content
255 was maximum (approximately, 3.3 mg/g of lyophilized product), whereas AYFYPEL was in low amounts or not
256 detected. The highest content of AYFYPEL (1.6 mg/g of lyophilized product) was obtained at 4 h and with the
257 lowest enzyme/substrate ratio. Fig. 3C shows the total amount of antihypertensive peptides in the hydrolysates,
258 from which it can be concluded that, the use of a higher enzyme/substrate ratio and the hydrolysis times between
259 6 and 10 h resulted in the hydrolysates with higher total content of these two active peptides.
260 With the aim of evaluating the type of substrate and the temperature of hydrolysate a second experiment
261 at laboratory scale was performed. Fig. 4 shows the estimated concentration of peptides RYLGY and AYFYPEL
262 in liquid hydrolysates of powdered milk products A (10 %, w/v) (Fig. 4a) and B (6 %, w/v) (Fig. 4b) for different
263 periods, 6 and 8 h, and temperatures of 37 and 50ºC. Again, the behavior observed in the formation of these two

peptides during hydrolysis was different. The concentration of RYLGY was higher by using powdered milk product A than product B, and in the hydrolysates obtained at 50ºC for 8 h. The concentration ranged from 29.1 μg/mL in powdered milk product B hydrolyzed for 6 h at 37ºC to 150.6 μg/mL in powdered milk product A hydrolyzed for 8 h at 50ºC. On the contrary, no changes were observed for the concentration of AYFYPEL between 6 and 8 h of hydrolysis at 37ºC; whereas hydrolysis at 50ºC produced lower values in the hydrolysates of both substrates. The concentration varied from 80.3 μg/mL, in powdered milk product A hydrolyzed for 8 h at 50ºC to 154.8 μg/mL in powdered milk product B hydrolyzed for 6h at 37ºC. Altogether, hydrolysis of powdered milk product A for 6 h at 50ºC produced relatively high amount of active peptides (198.1 μg/mL) being both peptides at a similar concentration (approx. 100 μg/mL).

Monitoring of peptides RYLGY and AYFYPEL in pilot scale hydrolysates

In order to study reproducibility of the hydrolysis process at larger volumes, hydrolysates of powdered milk product A were prepared at pilot scale by Innaves S.A. The hydrolysates were clarified, lyophilized, and, in some cases, ultrafiltered through 3 kDa membranes. The temperature was fixed to 45ºC. Other hydrolysis variables, concretely, substrate, enzyme/substrate ratio, range of enzyme addition and hydrolysis time, are described in Table 2. When the hydrolysates were prepared using powdered milk product A as substrate, the highest total content in peptides was obtained in hydrolysate nº 5 (Table 2), i.e., increasing the enzyme/substrate ratio and increasing hydrolysis time up to 24 h. However, this hydrolysate contained exclusively peptide RYLGY and the presence of both peptides is desirable to achieve a maintained antihypertensive effect, as demonstrated in animals [29]. Therefore, hydrolysates nº 7 and nº 9, manufactured with a high enzyme/substrate ratio for 12 h resulted in the hydrolysates with higher total amount of active peptides and containing at least 1 mg/g of peptide AYFYPEL. The enrichment of the powdered milk product A with casein did not improve the content of active peptides, as it can be seen by comparison of hydrolysates nº 7 and 9.

To evaluate ultrafiltration as strategy for enrichment in RYLGY and AYFYPEL, after a clarification step, hydrolysates nº 1 and 2 were ultrafiltered through a 3 kDa cut-off membrane. The 3 kDa-permeates were collected, and the ACE-inhibitory activity, expressed as IC_{50} value of the total hydrolysates and the permeates was measured. The ACE-inhibitory activity was high for both permeates, derived from hydrolysates nº 1 and 2, with IC_{50} values of 17.7 and 16.5 μg of protein/mL, respectively. The IC_{50} values of the respective total hydrolysates is at least twice higher (51.0 and 43.6 μg of protein/mL, respectively) indicating a lower ACE-inhibitory activity. However, the quantitative analysis of the peptides of interest showed that ultrafiltration was

not effective to improve the content in active peptides. The hydrolysates and the respective permeates contained similar peptide amounts (Table 2). Although IC_{50} is a good indicator to asses the ACE-inhibitory activity of individual peptides, it cannot be used to predict the amount of active peptides in complex mixtures, due to the interference with other active compounds [15, 38]. This highlights the need to quantify individual peptides by using a specific and accurate techniques.

Conclusions

Validation studies of the simple and rapid HPLC-MS method developed for quantitative determination of RYLGY and AYFYPEL showed sufficient specificity, reproducibility and linearity, demonstrating that this method can be used for the routine quantification of both peptides. Moreover, recovery of both peptides were greater than 80%. The monitoring of RYLGY and AYFYPEL during hydrolysis is essential to optimize the hydrolysis process and to evaluate techniques for peptide enrichment, such as ultrafiltration, and to optimize hydrolysis conditions. Some of the hydrolysates prepared at pilot scale contained the antihypertensive peptides in concentrations similar to that found in preparations with VPP and IPP used in clinical trials. For example, powdered milk product A at 20 % (w/v) hydrolyzed with pepsin during 12 h had a total content in active peptides of 3.66 mg/g.

These hydrolysates could be used as a nature ingredient in functional products to control blood pressure. Furthermore, extensive investigations are necessary in order to elucidate the minimal dose required of these peptides to show the antihypertensive effects in humans. In this context, a human trial with a yoghurt drink containing these peptides is already in progress.

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Figure captions

Fig. 1 (a) Total ion current (TIC) chromatogram of a selected powdered milk product hydrolyzed with pepsin and prepared at pilot scale, (b) extracted ion chromatogram (EIC) of the molecular ion of RYLGY *m/z* 671.2 and (c) EIC of the molecular ion of AYFYPEL *m/z* 902.3

Fig. 2 (a) Tandem mass spectrum of the singly charged ion *m/z* 671.2 and (b) of the singly charged ion *m/z* 902.3, that were identified as peptides RYLGY and AYFYPEL, respectively. The sequences of these peptides are displayed with the *b*- and *y*- type fragment ions observed in the spectra

Fig. 3 Content of peptides expressed as mg/g of dried product: (a) RYLGY, (b) AYFYPEL, and (c) sum of both peptides in peptic hydrolysates of powdered milk product A (10 %, w/v) at different times (4, 6, 8, 10 and 24 h) with low (—◇—) and high enzyme/substrate ratios (—■—) at 37ºC

Fig. 4 Concentration of RYLGY (▭) and AYFYPEL (■) expressed in μg/mL in liquid peptic hydrolysates of (a) powdered milk product A (10 %, w/v), and (b) powdered milk product B (6 %, w/v) obtained during 6 and 8 h and at 37 and 50 ºC. Protein content (—◇—) was determined by Kjeldhal method (%, w/v)

Table 1 Linear and polynomial regression for peak area vs. concentration of standard solutions of RYLGY and AYFYPEL in water obtained for HPLC–mass spectrometry (MS) and for HPLC–tandem mass spectrometry (MS/MS) using the ions indicated

Selected ions	Range (µg/mL)	n	Regression coefficients *a*	*b*	*c*	R^2	RSD	CV (%)
RYLGY								
MS (*m/z* 671.2)	1 to 20	10	---	3.114E6	---	0.999	0.951E6	4.2
	1 to 80	14	---	3.242E6	-1.513E4	0.996	3.500E6	6.6
MS (*m/z* 671.2, 693.2, 709.2)	1 to 20	10	---	3.847E6	---	0.996	2.491E6	8.6
	1 to 80	14	---	3.936E6	-2.233E4	0.994	4.820E6	7.9
MS/MS (*m/z* 654.2)	1 to 20	10	-1.152E5	2.000E5	---	0.996	0.944E5	7.0
	1 to 80	14	---	1.982E5	-1.075E3	0.980	4.641E5	15.2
MS/MS (*m/z* 274.9, 508.1, 654.2)	1 to 20	10	-1.315E5	2.760E5	---	0.997	1.072E5	5.7
	1 to 80	14	---	2.891E5	-1.624E3	0.989	4.951E5	11.3
AYFYPEL								
MS (*m/z* 902.3)	1 to 40	12	---	7.834E5	---	0.995	8.769E5	10.6
	1 to 80	14	-1.025E6	9.994E5	-5.534E3	0.998	6.804E5	5.4
MS (*m/z* 902.3, 924.3, 940.3)	1 to 40	12	---	1.377E6	---	0.987	2.567E6	16.8
	1 to 80	14	---	1.762E6	-1.360E4	0.995	1.608E6	7.6
MS/MS (*m/z* 357.9)	4 to 80	10	---	5.859E3	---	0.993	1.928E4	11.6
MS/MS (*m/z* 357.9, 521.2, 545.2, 771.2)	4 to 80	10	---	1.472E4	---	0.984	6.680E4	17.4
	4 to 80	10	-7.071E4	2.422E4	-0.141E3	0.989	4.012E4	10.4

Linear regression: $y = a + bx$ and polynomial regression: $y = a + bx + cx^2$; n: number of points; R^2: coefficient of determination; RSD: residual standard deviation; CV (%): residual standard deviation, expressed as a percentage of the mean value. All regression coefficients (*a*, *b*, *c*) presented are significantly different from zero ($P < 0.05$)

Table 2 Conditions of hydrolysis (substrate, enzyme/substrate ratio, incubation time and temperature) and content of peptides RYLGY and AYFYPEL in hydrolysates or 3 kDa fractions prepared at pilot scale

Hydrolysate nº	Lyophilized product type	Substrate (%, w/v)	Level of E/S ratio	t (h)	RYLGY content (mg/g)	AYFYPEL content (mg/g)	Total content (mg/g)
1	Hydrolysate	MPA (10)	Low	6	0.81 ± 0.03	1.47 ± 0.02	2.28 ± 0.01
2	Hydrolysate	MPA (10)	Low	8	1.10 ± 0.003	1.49 ± 0.11	2.59 ± 0.11
3	F< 3 kDa	MPA (10)	Low	6	0.86 ± 0.01	1.39 ± 0.03	2.25 ± 0.01
4	F< 3 kDa	MPA (10)	Low	8	0.93 ± 0.03	1.24 ± 0.05	2.17 ± 0.02
5	Hydrolysate	MPA (10)	High	24	3.98 ± 0.08	N.D.	3.98 ± 0.08
6	Hydrolysate	MPA (20)	Low	8	1.38 ± 0.04	1.37 ± 0.04	2.75 ± 0.04
7	Hydrolysate	MPA (20)	High	12	2.57 ± 0.10	1.09 ± 0.03	3.66 ± 0.13
8	Hydrolysate	MPA (20), CN (3)	Low	8	1.34 ± 0.02	1.52 ± 0.04	2.86 ± 0.07
9	Hydrolysate	MPA (20), CN (3)	High	12	2.65 ± 0.01	1.06 ± 0.04	3.71 ± 0.04
10	Hydrolysate	MPA (20), CN (3)	High	24	3.60 ± 0.10	N.D.	3.60 ± 0.10
11	Hydrolysate	MPA (30)	Low	8	0.95 ± 0.07	1.17 ± 0.16	2.12 ± 0.23
12	Hydrolysate	MPA (30), CN (3)	Low	8	0.80 ± 0.04	1.17 ± 0.01	1.97± 0.05

MPA: powdered milk product A; CN: casein; E/S ratio: enzyme/substrate ratio; N.D.: non detected.

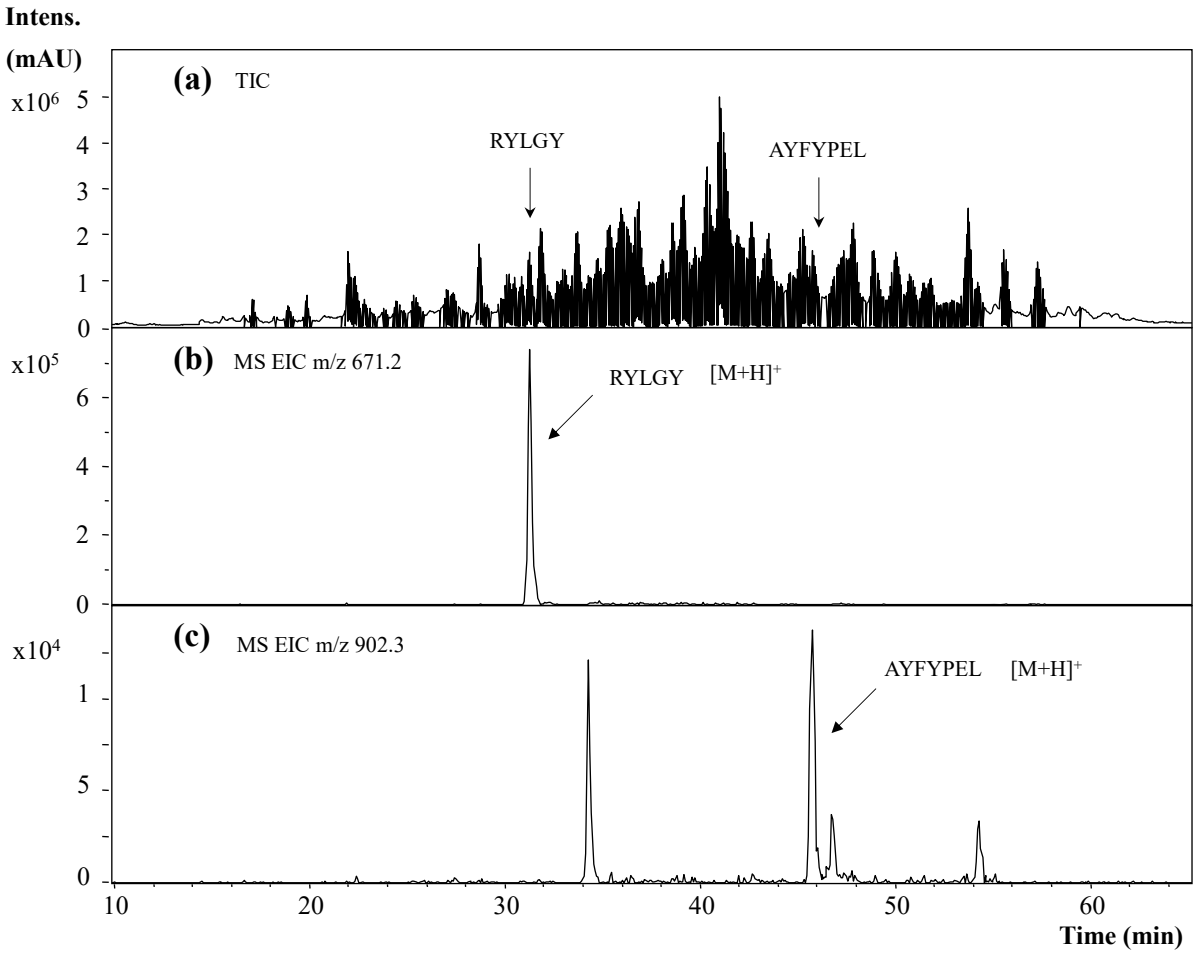

Intens.
(mAU)
x10⁶
(a) TIC
RYLGY
AYFYPEL
x10⁵
(b) MS EIC m/z 671.2
RYLGY [M+H]⁺
x10⁴
(c) MS EIC m/z 902.3
AYFYPEL [M+H]⁺
Time (min)

Fig. 1

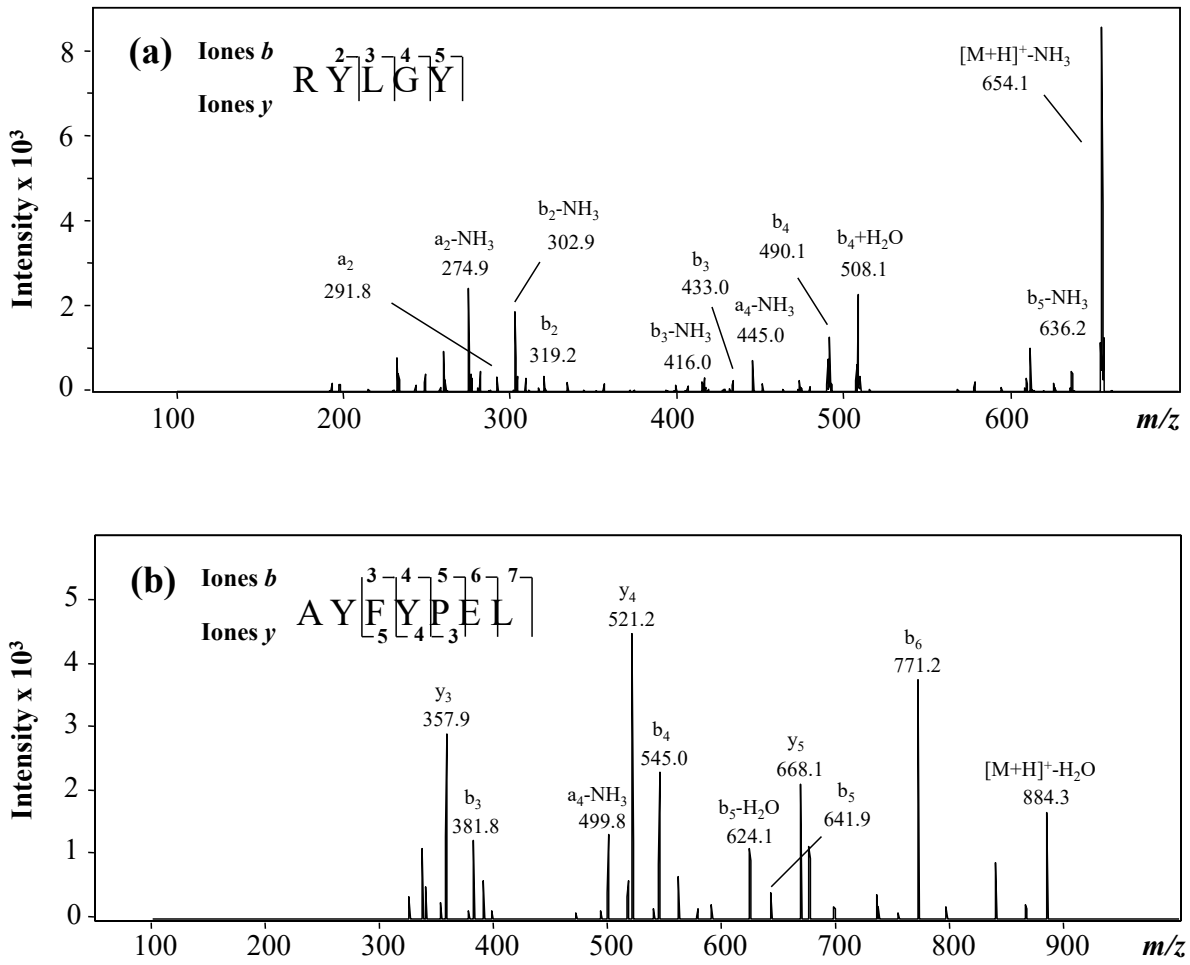
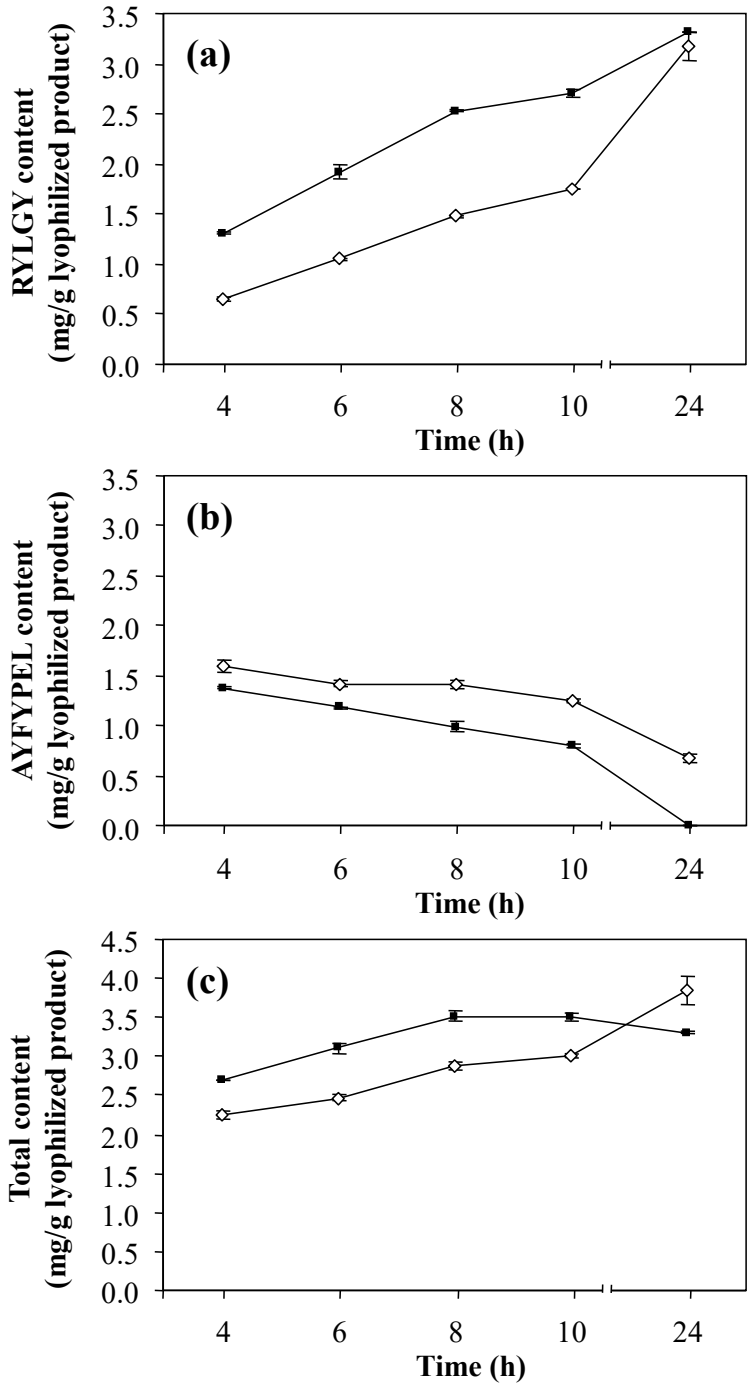

(a)
Ions b
Ions y
R Y L G Y
2 3 4 5
Intensity x 10³
m/z
[M+H]⁺-NH₃
654.1
b₂-NH₃
302.9
a₂-NH₃
274.9
a₂
291.8
b₂
319.2
b₃
433.0
b₃-NH₃
416.0
a₄-NH₃
445.0
b₄
490.1
b₄+H₂O
508.1
b₅-NH₃
636.2
(b)
Ions b
Ions y
A Y F Y P E L
3 4 5 6 7
5 4 3
Intensity x 10³
m/z
y₄
521.2
b₆
771.2
y₃
357.9
b₄
545.0
y₅
668.1
[M+H]⁺-H₂O
884.3
b₃
381.8
a₄-NH₃
499.8
b₅-H₂O
624.1
b₅
641.9

Fig. 2



431

432 **Fig. 3**

433

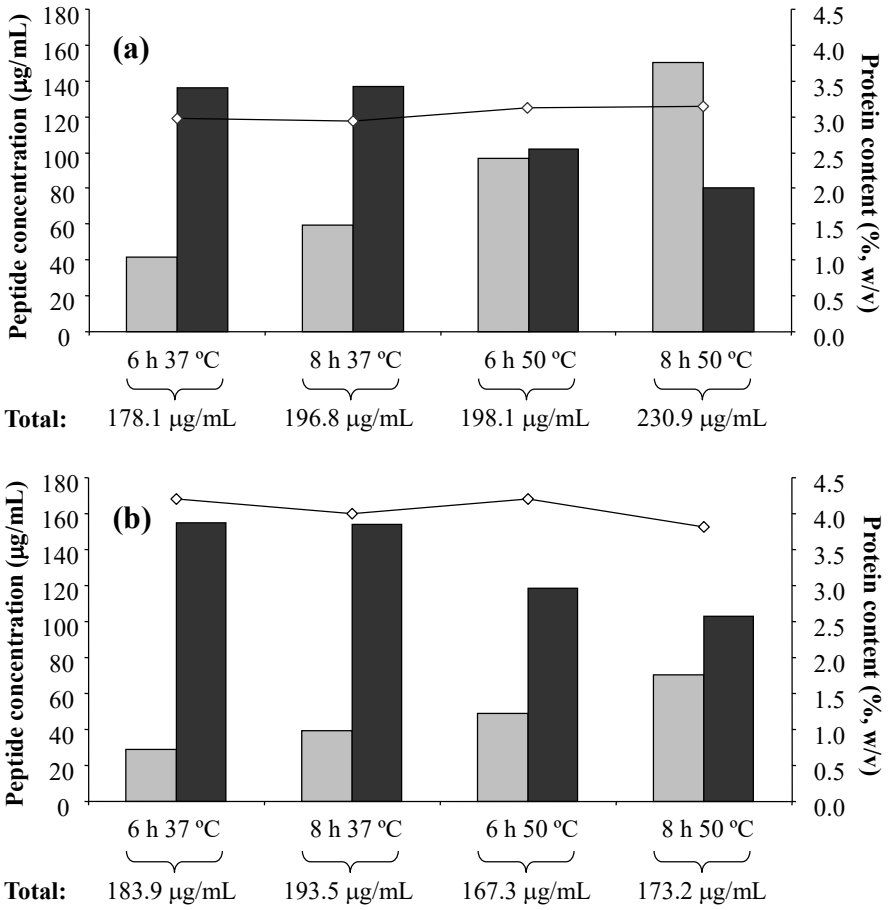

(a)
Peptide concentration (µg/mL)
Protein content (%, w/v)
180
160
140
120
100
80
60
40
20
0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
6 h 37 ºC
8 h 37 ºC
6 h 50 ºC
8 h 50 ºC
Total:
178.1 µg/mL
196.8 µg/mL
198.1 µg/mL
230.9 µg/mL
(b)
Peptide concentration (µg/mL)
Protein content (%, w/v)
6 h 37 ºC
8 h 37 ºC
6 h 50 ºC
8 h 50 ºC
Total:
183.9 µg/mL
193.5 µg/mL
167.3 µg/mL
173.2 µg/mL

Fig. 4