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β-lactoglobulin peptides obtained by chymotrypsin hydrolysis

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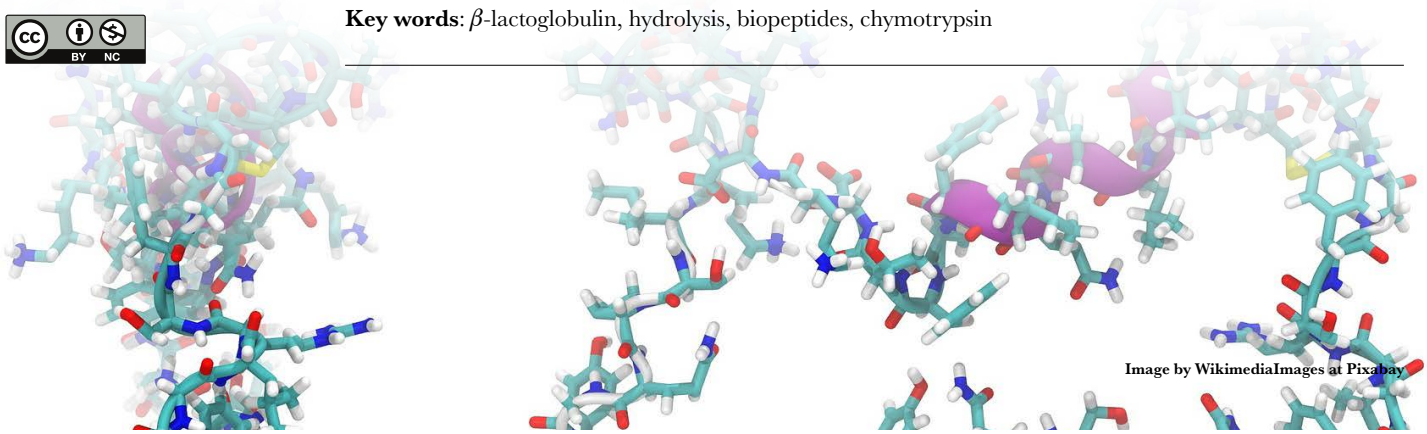
ABSTRACT

Objective: Whey proteins, as β-lactoglobulin, have biological activity. Controlled hydrolysis of this protein could generate peptides with some biological function. The aim of this work was to analyze the peptides resulting from the *in vitro* hydrolysis with chymotrypsin in order to evaluate the presence of bioactive peptides.
Design/methodology/approach: Chymotrypsin was used in the hydrolysis of β-lactoglobulin, and its peptides were evaluated by ultrafiltration, electrophoresis, and mass spectrometry.
Findings/conclusion: Results showed that 2 h of chymotrypsin hydrolysis (T1) released peptides with molecular weight values of 8 and 9 KDa, while 4 h of hydrolysis (T2) produced peptides with molecular mass weight values of 7 and 5 KDa. The mass spectrometry (MALDI-TOF) showed six peaks and five of them were comparable with those obtained by *in silico* hydrolysis results (done previously by Fonseca Ayala, 2018). The identified peptides (DTDYK, DAQSAPL and LKPTPEGDL) in the fraction <1 kDa showed inhibitory activity of angiotensin converting enzyme and inhibitory activity of enzyme dipeptidyl peptidase IV according BIOPEP database. These results showed that β-lactoglobulin peptides obtained by chymotrypsin hydrolysis could have biological activity that can be used in different types of industries as pharmaceutical and food.
Limitations on study/implications: The *in vitro* evaluation of the biological activity of the characterized peptides is necessary.

Key words: β-lactoglobulin, hydrolysis, biopeptides, chymotrypsin



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INTRODUCTION

Bioactive peptides are protein fragments that have a positive effect on body functions and health (Remanan and Wu, 2014; Sharma *et al.*, 2011), this fact has increased their scientific and commercial interest (Korhonen, 2009). These peptides are inactive within the protein, but they can be released during enzymatic hydrolysis under specific conditions in the gastrointestinal tract, therefore enzymes as pepsin, trypsin and chymotrypsin have been used. Antimicrobial, antithrombotic, antihypertensive, opioid, immunomodulatory, and antioxidant activities had been described and such activities depends on their amino acids sequence and composition (Escudero *et al.*, 2012).

β-Lactoglobulin is the main protein in whey, it has antioxidant activity and maintains retinol absorption levels in dermal cells, it also has emulsifying capacity derived from its amphiphilic structure (Pihlanto-Leppälä, 2000). Besides, bioactive peptides of this protein with different biological activities have been described such as ACE inhibitory activity (Hernández-Ledesma *et al.*, 2011; Pihlanto-Leppälä, 2000), dipeptidyl peptidase IV inhibitory activity (Girolamo *et al.*, 2008) antimicrobial capacity (Mulero *et al.*, 2011), and some peptides had been used with others showed a positive effect on metabolic syndrome (Ricci-Cabello *et al.*, 2012).

Recently, Fonseca Ayala (2018) determined the bioactivity of peptides obtained by *in silico* hydrolysis of β-Lactoglobulin with different enzymes (chymotrypsin, trypsin and pepsin) where 29 peptides were obtained by chymotrypsin hydrolysis and three of them have biological activity according BIOPEP database. Their results show that chymotrypsin hydrolysis *in vitro* could produce bioactive peptides. Therefore, the objectives of this work were to do the *in vitro* hydrolysis of β-lactoglobulin with chymotrypsin and to compare the produced peptides with those obtained by *in silico* hydrolysis.

MATERIALS AND METHODS

Materials

Bovine lyophilized β-lactoglobulin (purity >/=90%) (L3908 Sigma-Aldrich), and β-chymotrypsin from bovine pancreas type II (C4129 Sigma-Aldrich). All materials were reagent grade.

Methods

Chymotrypsin hydrolysis. The hydrolysis was carried out according to Gillespie *et al.* (2015) with some modifications. 0.2 g of β-lactoglobulin was added to 25 ml of phosphates buffer pH 7.4 after, chymotrypsin was added in a 1:25 proportion enzyme/substrate, the reaction was done at 37 °C in agitation. Three hydrolyzed solutions were obtained, T0: without reaction time, T1: 2 h of reaction, T2: 4 h of reaction. The inactivation of the enzyme was carried out at 90 °C during 2 min. The hydrolyzed solutions were stored at freezing (−20 °C) until their analysis.

Degree of hydrolysis. A pH method was carried out where the samples were titrated with NaOH (0.1N) in order to maintain the optimal pH of the enzyme. The degree of hydrolysis (%DH) were calculated with Equation 1 (Adler-Nissen, 1979; Fernández and Kelly, 2016).

$$DH\,(\%) = \left[\frac{\beta \times Nb}{\alpha \times Mp \times Htot}\right] \times 100 \qquad \text{[Eq.1]}$$

where: β: (mL) volume of the NaOH solution added during the hydrolysis. *Nb*: (eq/L) is the normality of the NaOH solution. *Mp*: g of the protein in the reaction. *Htot*: (mequiv/g) total number of peptide bonds in the substrate. α: degree of dissociation of β-amino groups released during the hydrolysis. Both, *Htot* (8.8) and α (1) values were obtained from the literature (Nielsen *et al.*, 2001).

Ultrafiltration of the hydrolyzed solutions. The hydrolyzed solutions were ultrafiltrated using a shaking chamber (Amicon 8010 Millipore) with a capacity of 10mL and a membrane filtration of 4.1 cm^2 of area (regenerated cellulose, Ultracel®). Membranes had a molecular scale of 5, 3 and 1KDa and were hydrated in distilled water for 2 h before the ultrafiltration. The separation was carried out at a pressure of 25 psi with N_2 and at a temperature of 25 °C, and in three stages: 1) The hydrolyzed solution (T1, T2) (10 mL) was put into the chamber with a 5 KDa membrane and the permeated solution was obtained, 2) this permeated solution (5 KDa) was put into the chamber with a 3 KDa membrane and the permeated solution was recovered, 3) the second permeated solution (3 KDa) was put into the chamber with a 1 KDa membrane. All permeated solutions were store at freezing (−20 °C) until their analysis.

SDS-PAGE/ Tricine polyacrylamide Gel Electrophoresis. a) Pre-electrophoresis sample preparation: 10 μL of sample [BSA; β-LG; 1 KDa permeated solution and unfiltered hydrolyzed solutions (T0, T1, T2) were added to 25 μL of buffer (TruPAGE 4x PCG3009, Sigma Aldrich) with 65 μL of distilled water. The mixture was stirred for 5 s until homogenized and heated at 70 °C during 10 min and cooled to −5 °C during 10 min before the analysis. The markers (Sigma S8445-10VL) (200000-6500 KDa) were prepared according to the manufacturer. b) SDS-PAGE/Tricine polyacrylamide gel Electrophoresis: The samples were analyzed by electrophoresis using a Mighty Small II SE 250 camera (Hoefer Scientific Instruments). A precast 20% to 4% gradient gel with 12 wells was used. The electrophoretic run was carried out at a voltage of 100 volts during 2 h. 10 μL of the samples and the marker were injected into each well of the gel.

Molecular weights values of the peptides of permeated solution (<1 KDa) by Mass Espectrometry (MALDI-TOF / TOF). The Autoflex Speed equipment (Bruker Daltonics) was used. The permeated solution <1 KDa of both chymotrypsin hydrolyzed solutions (T1 and T2) were dissolved in a 0.1% trifluoroacetic acid solution with three parts of acetonitrile. This mixture was prepared with a 1:1 matrix: sample ratio. The matrix was alpha-cyano-4-hydroxycinnamic acid (Sigma Aldrich). The analysis of the permeated solutions was carried out in the range between 700 and 3500 Da. The identified peaks (peptides) in the spectrogram were compared to the molecular weights values obtained by *in-silico* hydrolysis carried out by Fonseca Ayala (2018). After that, the possible bioactivity of these peptides was identified in the BIOPEP database.

RESULTS AND DISCUSSION

The chymotrypsin hydrolysis and evaluation of the degree of hydrolysis (DH) by pH-stat method were carried out simultaneously, results (Table 1) show that the DH was higher at 4 h (T2) (25.5%) than 2 h (T1) (22%), and these values are according to Tulipano *et al.* (2015) who obtained similar DH values. pH-stat method measures the protons released from the active site of chymotrypsin by their catalytic activity and indirectly determines the cleavage of the peptide bond by the number of amino groups released.

After hydrolysis, the fractions were analyzed by SDS-PAGE gel electrophoresis, the results (Figure 1 and Table 2) show that hydrolyzed fractions had different molecular

Table 1. Degree of Hydrolysis after 2 (T1) and 4(T2) h of hydrolysis using chymotrypsin.

Degree Hydrolysis (%DH)		
T0	**T1**	**T2**
0	22 %	25.52%

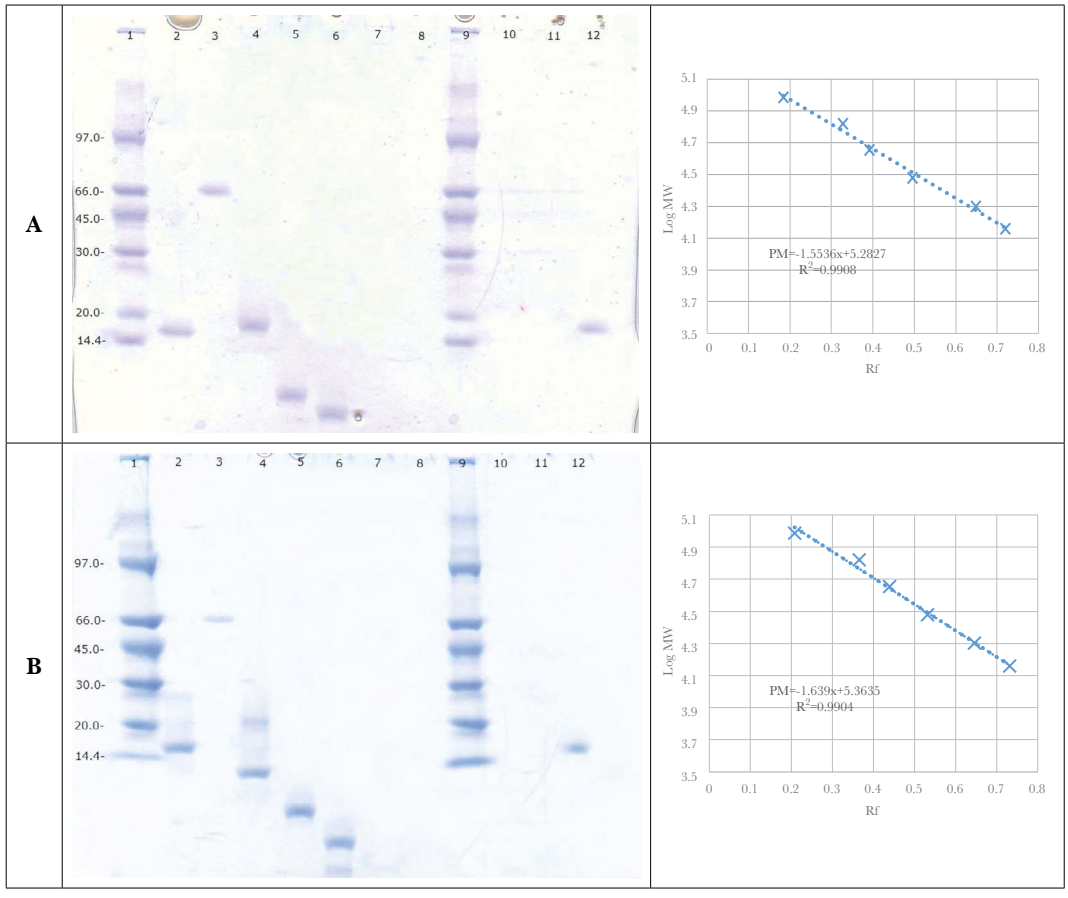

Figure 1. Images of electrophoresis gels of different fractions after chymotrypsin hydrolysis (A) and linear regression of the Rf *vs* Log molecular weight (LogMW) (B). 1) Low spectrum standard (97.0-14.4 KDa); 2) β-Lg (1mg/mL); 3) BSA (1mg/ml); 4) T0; 5) T1; 6) T2; 7) Permeated solution <1 KDa of T1 8); Permeated solution <1 KDa of T2; 9) Low spectrum standard (97.0-14.4 KDa); 10) Permeated solution <1 KDa of T1; 11) Permeated solution <1 KDa of T2; 12) β-Lg (1mg/L).

Table 2. Molecular weight values identified by the linear regression analysis of Rf and Log WM of electrophoresis gel of chymotrypsin hydrolyzed fractions.

Samples	Gel 1				Gel 2	
	Rf	Log PM	PM(Da)	Rf	Log PM	PM(Da)
β-Lg	0.696	4.2014	15899.90	0.646	4.2713	18676.69
BSA	0.328	4.7731	59308.81	0.3	4.8563	71829.03
T0	0.688	4.2138	16361.50	0.712	4.1608	14481.05
T1	0.864	3.9404	8717.45	0.815	3.9853	9667.18
T2	0.92	3.8534	7134.90	0.9	3.8423	6955.046
T2.b				0.96	3.25	4950.365

weight values, T0 had values around 16 or 14 KDa similar to the molecular weight of β-Lg, which molecular weight was 15 KDa (Gel 1) and 18 KDa (Gel 2). T1 had fractions of 8 KDa (Gel 1) and 9 KDa (Gel 2), while T2 had fractions of 7 KDa (Gel 1 and 2) and 5 KDa (Gel 2). Also, a fraction <1 KDa was analyzed, but no bands were presented because of the gel sensibility. These results showed that as the chymotrypsin hydrolysis time increased hydrolyzed fractions had lower molecular weight values.

The portions <1 KDa of both hydrolyzed fractions (T1 and T2) were analyzed in the mass spectrometer in order to know the molecular weight of their peptides, however only the spectrogram of T1 had six identified peaks (Table 3). These molecular weight values were compared with the peptides obtained by *in silico* hydrolysis (Fonseca Ayala, 2018) but only five peptides were comparable with them. Also, three peptides (2, 5 and 6) could have bioactivity as ACE inhibitory activity and Dipeptidyl peptidase IV inhibitory activity and peptides 2, 4 and 6 could have antibacterial activity, because similar sequences were described in the BIOPEP database. However, the *in vitro* analysis of the biological activity of these peptides is necessary to corroborate these results.

Table 3. Molecular weight values of T1 identified by mass spectroscopy and its sequence identified by *in silico* hydrolysis.

No.	Molecular weight values of T1	Sequence	Sequence identified in BIOPEP database	Bioactivity reported in the BIOPEP database
1	620.3 Da			
2	643.22 Da	DTDYK	VLDTDYK	ACE inhibitor
			VLVLDTDYK	Antibacterial
			VLVLDTDYK	Dipeptidyl peptidase IV inhibitor
3	656.85 Da	EEQCH		
4	686.74 Da	AASDISL	AASDISLLDAQSAPLR	Antibacterial
5	716.80 Da	DAQSAPL	LDAQSAPLR	ACE inhibitor
			AASDISLLDAQSAPLR	Antibacterial
			DAQSAPLRVY	ACE inhibitor
6	742.70 Da	LKPTPEGDL	LKPTPEGDL	Dipeptidyl peptidase IV inhibitor
			LKPTPEGDLEIL	Dipeptidyl peptidase IV inhibitor

CONCLUSIONS

Chymotrypsin hydrolysis produced fractions of 8 KDa and 9 KDa at 2 h, and 7 KDa and 5 KDa at 4 h of hydrolysis. Mass spectrometry identified six peptides in the hydrolyzed solution (T1, 2h), where five of them were comparable with peptides previously obtained by *in silico* hydrolysis. The sequences of the peptides identified (DTDYK, DAQSAPL and LKPTPEGDL) showed bioactivity according BIOPEP database.

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