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Effect of simulated gastrointestinal digestion on the antihypertensive properties of synthetic β-lactoglobulin peptide sequences

Blanca Hernández-Ledesma[1], Marta Miguel[1], Lourdes Amigo[1], Maria Amaya Aleixandre[2] and Isidra Recio[1]*

[1] Instituto de Fermentaciones Industriales (CSIC). Madrid. Spain
[2] Instituto de Farmacología y Toxicología (CSIC). Departamento de Farmacología, Facultad de Medicina, Univ. Complutense, Madrid, Spain

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In this study, the antihypertensive activity in spontaneously hypertensive rats of two peptides isolated from β-lactoglobulin hydrolysates with thermolysin was evaluated. These peptides, with sequences LLF [β-lg f(103–105)] and LQKW [β-lg f(58–61)], showed potent *in vitro* ACE-inhibitory activity. Two hours after administration, both sequences caused a clear and significant decrease in the blood pressure of these rats. The impact of a simulated gastrointestinal digestion on ACE-inhibitory and antihypertensive activities of these peptides was also studied. The results showed that both fragments were susceptible to proteolytic degradation after incubation with pepsin and Corolase PP®. In addition, their *in vitro* ACE-inhibitory activity decreased after the simulated digestion. It is likely that fragment LQK was the active end product of the gastrointestinal digestion of peptide LQKW. The fragment LL, observed after digestion of peptide LLF, probably exert its antihypertensive effect through a mechanism of action different than ACE-inhibition.

Keywords: β-lactoglobulin peptides, ACE-inhibitory activity, antihypertensive effect, simulated gastrointestinal digestion.

It is accepted that enzymatic hydrolysis of major whey proteins, β-lactoglobulin (β-lg) and α-lactoglobulin (α-la) releases peptides that may exhibit different biological activities. Among the different groups of bioactive peptides, angiotensin-converting enzyme (ACE)-inhibitory peptides have been extensively studied due to their potential effects in the prevention of hypertension. These peptides could also be administered together with antihypertensive drugs to improve the pharmacological treatment of this disease (Fitzgerald & Meisel, 2000). Recently, a potent antihypertensive effect has been reported for a whey protein concentrate hydrolysed with Alcalase (Costa et al. 2005). These authors have suggested a pathway involving ACE inhibition as mainly responsible of this effect. Several ACE-inhibitory and antihypertensive peptides have been isolated and characterised from α-la and β-lg hydrolysed with digestive enzymes (Pihlanto-Leppälä et al. 2000; Sipola et al. 2002; Chobert et al. 2005). In our laboratory, two potent ACE-inhibitory peptides derived from caprine β-lg were identified (Hernández-Ledesma et al. 2002). These peptides, with the sequences LQKW and LLF, were released from the whey protein after incubation with thermolysin at 37 °C for 24 h. Increasing the incubation temperature up to 60 °C enables the first of these two peptides to be released from bovine β-lg after 20 min incubation with this enzyme (Hernández-Ledesma et al. 2006).

The aim of this work was to evaluate the antihypertensive activity of β-lg peptides LQKW and LLF in spontaneously hypertensive rats. Moreover, the behaviour of these peptides under simulated gastrointestinal digestion conditions was also studied.

Material and Methods

Measurement of ACE-inhibitory activity

Synthetic peptides derived from β-lg, with sequences LQKW and LLF, were synthesized by GenScript Corporation (Piscataway, NJ, 08854, USA). ACE-inhibitory activity was measured by the spectrophotometric assay of Cushman & Cheung (1971), with some modifications. Briefly, 40 µl of each sample was added to 0·1 ml 0·1 M-sodium borate

*For correspondence; e-mail: recio@ifi.csic.es

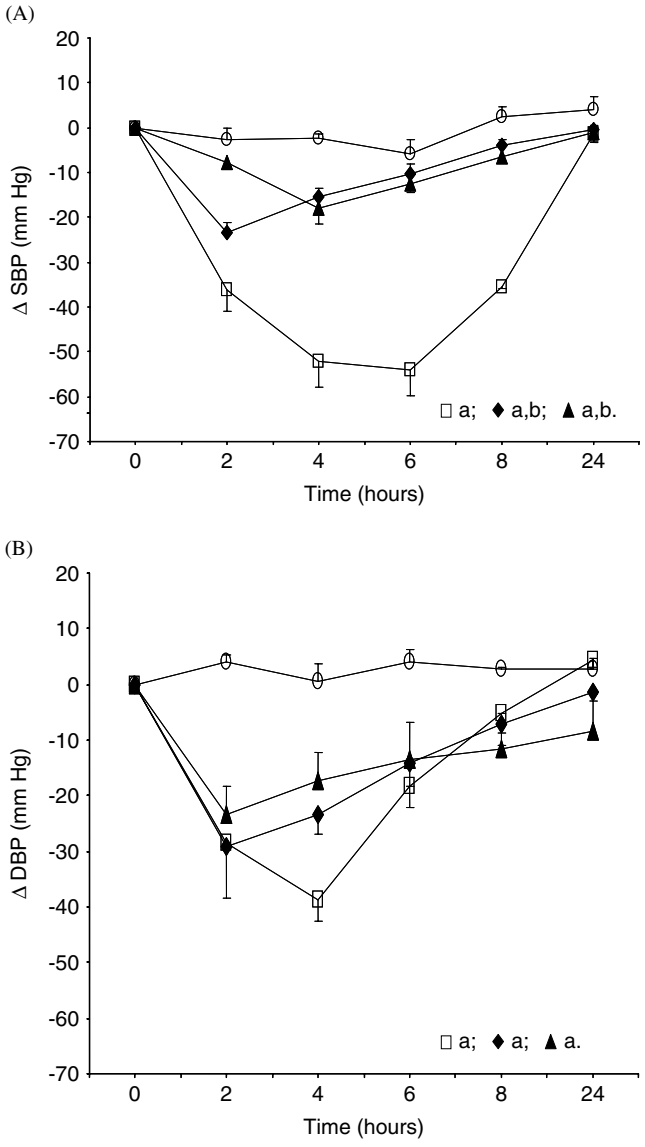

Fig. 1. (A) Decrease in systolic blood pressure (SBP) and (B) in diastolic blood pressure (DBP) caused in spontaneously hypertensive rats by the administration of water (○), Captopril (50 mg/kg) (□) and 10 mg/kg of two peptide sequences isolated from β-lactoglobulin hydrolysates: LLF (◆) and LQKW (▲). The data represent the mean values ± SEM for a minimum of 5 rats. Values significantly different ($P<0{\cdot}05$) from a, water or b, Captopril are indicated.

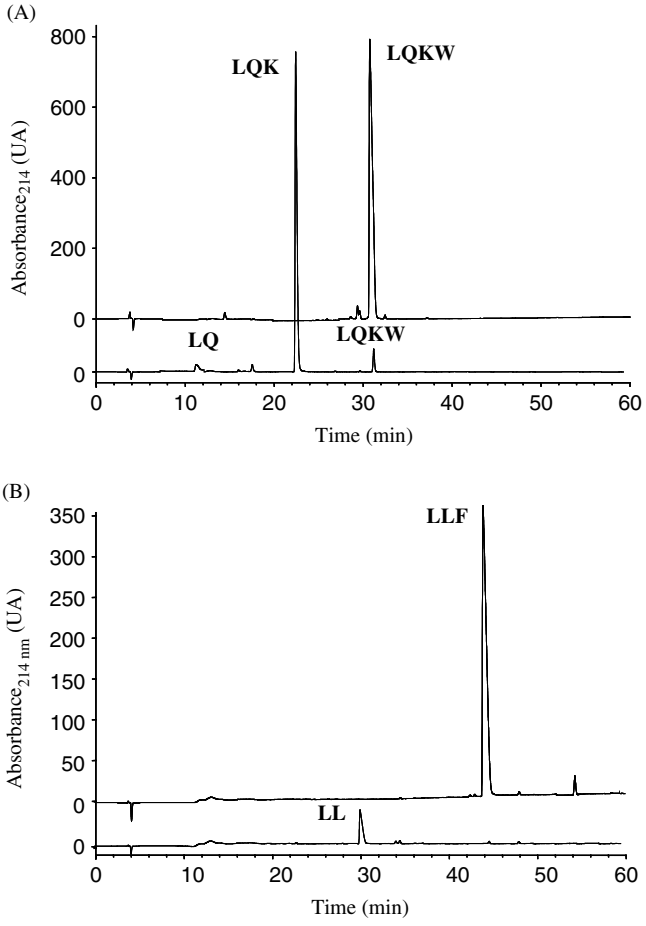

Fig. 2. UV-chromatograms of the β-lactoglobulin peptides (A) LQKW and (B) LLF, before and after simulated physiological digestion.

buffer (pH 8·3) containing 0·3 M-NaCl, and 5 mM-hippuryl-histydil-leucine (Sigma Chemical, St. Louis, MO, 63103, USA). ACE (2 mU; EC 3.4.15.1, 5·1 U mg^{-1}, Sigma) was added and the reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 0·15 ml 1 M-HCl. The hippuric acid formed was extracted with ethyl acetate, heat-evaporated at 95 °C for 10 min, redissolved in distilled water and measured spectrophotometrically at 228 nm. The activity of each sample was tested in triplicate. ACE-inhibitory activity was calculated as the protein concentration required to cause 50% inhibition of the original ACE activity (IC_{50}).

Measurement of antihypertensive activity

Male spontaneously hypertensive rats (SHR), 17 to 20 weeks old, weighing 300–350 g (Charles River Laboratories Spain S.A., Spain) were used in this study. The animals were maintained at a temperature of 23 °C with 12 h light/dark cycles and consumed tap water and a standard diet for rats (A04 Panlab, Barcelona, 08940, Spain) *ad libitum* during the experiments. The peptide sequences LQKW and LLF were orally administered to the animals. Only one dose of 10 mg/kg body weight of these sequences was administered by gastric intubation between 9 and 10 a.m. Distilled water served as negative control, and Captopril (50 mg/kg; Sigma, USA), a known ACE inhibitor, served as positive control in the experiments. All the products were administered in 1 ml water. The systolic blood pressure (SBP) and the diastolic blood pressure (DBP) of the rats were measured by the tail cuff method (Miguel et al. 2005),

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Table 1. Peptide fragments released and ACE-inhibitory activity after simulated gastrointestinal digestion of the synthetic β-lg derived peptides

	IC_{50} (μg/ml)†			
Sequence	Before digestion	After digestion	Degree of hydrolysis	Fragments released after digestion
LQKW	2·0 (3·5 μM)	54·2	Partially hydrolysed	LQKW; LQK; LQ
LLF	32·2 (82·4 μM)	>1500	Hydrolysed	LL

†Concentration of peptide needed to inhibit the original ACE activity by 50%

before administration and also 2, 4, 6, 8, and 24 h post-administration using a LE 5001 equipment (Letica, Barcelona, 08940, Spain). To compare the different treatments, the data were analysed by a two-way analysis of variance (ANOVA), using the Graph Pad Prism 4 software. Differences between the means were considered to be significant when $P<0·05$. All the above-mentioned experiments were performed as authorised for scientific research (European Directive 86/609/CEE and Royal Decree 223/1988 of the Spanish Ministry of Agriculture, Fisheries and Food).

Simulation of gastrointestinal digestion

Simulation of gastrointestinal digestion of peptides was carried out according to the method proposed by Alting et al. (1997) and modified by Quirós et al. (2005). Briefly, peptides dissolved in water (0·7% wt protein/vol) were hydrolyzed with pepsin (20 mg/g peptide; EC 3.4.23.1; 1 : 10 000, 890 U/mg protein; Sigma) for 90 min at 37 °C at pH 2. Then, the pH was adjusted to 7·5 with 1 M-NaOH, Corolase PP® was added (40 mg/g protein), and the samples were incubated for 150 min at 37 °C. The enzyme was inactivated by heating at 95 °C for 15 min, followed by cooling to room temperature. Hydrolysates were centrifuged at 35 000× ***g*** for 30 min and the supernatants were stored at −20 °C until further analysis.

Analysis by on line RP-HPLC-MS/MS

The peptides and the digests obtained after their hydrolysis with pepsin and Corolase PP® were analysed by RP-HPLC-MS/MS on an Agilent HPLC system connected on line to an Esquire-LC quadrupole ion trap instrument (Bruker Daltonik GmbH, D-28359 Bremen, Germany), following the method proposed by Hernández-Ledesma et al. (2004). Peptides were eluted with a linear gradient of 0% to 45% solvent B over 60 min at a flow rate of 0·8 ml/min. Mass spectra were acquired over the range 50–1500 *m/z* (depending on the *m/z* and the charge state of the precursor ion). Using Data Analysis™ (version 3.0; Bruker Daltoniks), the *m/z* spectral data were processed and transformed to spectra representing mass values. BioTools (version 2.1; Bruker Daltoniks) was used to process the MS(n) spectra and to perform peptide sequencing.

Results and Discussion

ACE-inhibitory and antihypertensive activities of synthetic β-lg peptides

The peptides LQKW and LLF, which had been previously identified in hydrolysate of caprine β-lg with thermolysin (Hernández-Ledesma et al. 2002), were synthesised and their ACE-inhibitory activity was measured. This activity was calculated as IC_{50}, that is, the protein concentration required to inhibit the original ACE activity by 50%. These peptides showed a potent ACE-inhibitory activity, with IC_{50} values of 3·5 and 82·4 μM, respectively. The IC_{50} measured for peptide LQKW was 10-fold lower than that previously reported by Hernández-Ledesma et al. (2002) (IC_{50} of 34·7 μM). Probably, the different procedure used for synthesizing the peptide in the present study could lead to the formation of more active conformers. Gómez-Ruiz et al. (2004) also attributed the differences found in the ACE-inhibitory activity of two preparations of peptide DKIHP to the method used for synthesizing them and the subsequent presence of *trans*- or *cis*-conformers with different activities.

In order to investigate whether the studied sequences induced any changes in arterial blood pressure, we also measured this variable in SHR rats. Both sequences caused a clear and significant decrease in the SBP and the DBP of these rats ($P<0·05$; Fig. 1). No differences were observed between LLF and LQKW. The maximum decreases in SBP (∼20 mmHg) were at 2 and 4 h after administration, respectively (Fig. 1A). The greatest decrease in SBP was observed 4–6 h after administration of Captopril. However, the decrease in DBP caused by these peptides was very similar to the decrease in this variable caused by Captopril ($P>0·05$), with maximum values for LLF and LKQW after 2 h administration (∼25 mmHg; Fig. 1B).

Simulated gastrointestinal digestion: peptides released and ACE-inhibitory activity

In order to simulate gastrointestinal digestion conditions, the synthetic peptides were subjected to a two-stage hydrolysis process, which simulates gastrointestinal digestion conditions. With the aim of identifying the fragments released by the action of digestive enzymes, the hydrolysates were analysed by HPLC-MS/MS. Figure 2A & B show the

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RP-HPLC chromatograms of peptides LQKW and LLF, respectively, before and after simulated gastrointestinal digestion. The peptide LQKW was partially hydrolysed by digestive enzymes, thus a small amount of this peptide could be detected in the final hydrolysate. However, the main constituent of this hydrolysate was the fragment LQK, released after action of the enzymes on the C-terminal residue of peptide LQKW (Fig. 2A). A small amount of peptide LQ could also be observed in the final hydrolysate. A moderate decrease in ACE-inhibitory activity was observed after simulated digestion. However, the IC_{50} value of the final digest (54·2 μg/ml, Table 1) was still low compared with the IC_{50} values reported for other milk and egg-proteins hydrolysed under similar simulated gastrointestinal conditions (Quirós et al. 2005; Miguel et al. 2006). The main fragment released during hydrolysis, LQK, was synthesized and its ACE-inhibitory activity was measured, showing an IC_{50} value of 55·3 μg/ml. This result indicated that this peptide could be responsible for the ACE-inhibitory activity observed in the final hydrolysate. Therefore, if peptide LQKW is degraded *in vivo* following the same hydrolysis pattern, the fragment LQK would be responsible for the high antihypertensive activity measured when peptide LQKW was administered to SHR.

The behaviour of peptide LLF after hydrolysis with pepsin and Corolase PP can be observed in Fig. 2B. This peptide is included within a region of β-lg, which had been considered as a 'strategic zone' of protein, partially protected from digested breakdown. However, when this peptide was subjected to a simulated gastrointestinal digestion, the digestive enzymes acted on the C-terminal residue and a small amount of fragment LL was released, and was the only fragment observed in the final digest. The final digest did not show ACE-inhibitory activity, presenting an IC_{50} value higher than 1500 μg/ml (Table 1). However, an important antihypertensive effect could be observed when peptide LLF was orally administered to SHR. It is important to note that other mechanisms of action different from ACE inhibition could also be involved in the blood pressure lowering effect exerted by this peptide. The sequence LLF is included within the sequence of opioid peptide β-lactorphin. This peptide improves the endothelium-dependent relaxation to acetylcholine in mesenteric arterial preparations of SHR. This effect was partially mediated by nitric oxide. Moreover, the sequence YLLF also enhances the endothelium-independent relaxation induced by sodium nitroprusside in these arteries (Sipola et al. 2002). Further research is therefore needed to clarify the mechanism/s involved in the antihypertensive effect of the studied peptides.

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