Peptide fragments from β-casein f(134-138), HLPLP, generated by the action of rat blood plasma peptidases show potent antihypertensive activity

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ABSTRACT

The intact absorption of a β-casein peptide, HLPLP, into rat blood circulation after oral administration was recently demonstrated. In addition to the parent peptide, several derived fragments were detected in rat plasma. The aim of the present work is to elucidate whether these fragments retain antihypertensive activity and if their activity is mediated by angiotensin-converting-enzyme inhibition. The penta-peptide was incubated in rat plasma in order to identify and quantify the fragments generated by the action of plasmatic peptidases on HLPLP, using tandem mass spectrometry. Peptides HLPL, LPLP and HLP were generated within seconds of incubation. The parent peptide, HLPLP, and all possible derived peptides showed potent antihypertensive activity in spontaneously hypertensive rats and caused inhibition of vascular contraction elicited by angiotensin-I. It can be concluded that the antihypertensive effect of HLPLP can be produced by the concomitant action of the parent peptide and several novel derived fragments.

Keywords: Antihypertensive peptides/ in vivo active form/ plasma peptidases/ SHR/ tandem mass spectrometry.
1. Introduction

In recent years, the evaluation of changes that bioactive peptides undergo in the organism after ingestion has gained importance, since the administered form and the active form can be different. The identification of the *in vivo* active form will allow a better establishment of the mechanism of action, and of the cause/effect relationship for bioactive peptides, which are needed to support a health claim. These changes take place during gastrointestinal digestion and subsequent absorption, distribution, metabolism and elimination (ADME). Upon gastrointestinal digestion, food peptides can resist or can undergo hydrolysis, and this may cause a loss of their activity or, on the contrary, the release of the active sequence. For instance, the active form of the peptide β-casein f(169-175) is generated after losing the C-terminal glutamine residue during intestinal digestion (Maeno, Yamamoto, & Takano, 1996). Similarly, an antihypertensive peptide from a casein hydrolysate, αs1-casein f(90-94) RYLGY, was partly degraded when subjected to simulated gastrointestinal digestion, giving rise to smaller peptides. Some of these peptide fragments thereof, RYLG, RY, and YLGY retained antihypertensive activity while YLG did not exert any antihypertensive effect (Contreras, Sánchez, Sevilla, Recio, & Amigo, 2013).

Once the stability of peptides to digestion is ensured, bioavailability studies need to be conducted, in order to determine the accessibility of potential biologically active peptides in the organism (Foltz, van der Pijl, & Duchateau, 2010; Picariello et al., 2013). The assessment of the peptide stability to soluble plasma peptidases once they are absorbed can play an important role on the exhibition of its biological effect (Vermeirssen, Van Camp, & Verstraete, 2004; Miner-Williams, Stevens, Moughan, 2014). In this regard, mass spectrometry
(MS)-based techniques have permitted the development of sensitive methods to detect and quantify small changes of peptides in plasma samples, reaching detection limits in the picogram range (van Platerink, Janssen, Horsten & Haverkamp, 2006). In addition, these techniques allow to follow the peptide biotransformations which can take place in the organism.

The peptide LHLPLP, f(133-138) from β-casein, present in an antihypertensive fermented milk, was identified as one of the sequences that most contributed to the biological effect caused by this fermented milk, and this product exerted angiotensin-converting-enzyme (ACE)-inhibitory and antihypertensive activity after acute and long-term oral administration (Quirós et al., 2007; Miguel et al., 2005). This sequence was reported to be resistant to gastrointestinal digestion (Quirós, Contreras, Ramos, Amigo & Recio, 2009). However, when incubated with Caco-2 cells, the hexapeptide was partly degraded by brush border-like enzymes expressed by these cells, releasing the peptide HLPLP, f(134-138), which underwent epithelial transport across the Caco-2 cell monolayer (Quirós, Dávalos, Lasunción, Ramos & Recio, 2008). Recently, β-casein f(133-138), HLPLP has been found in rat blood circulation after its oral administration at 40 mg/kg body weight (absorption ratio of 5.18%) (Sánchez-Rivera et al., 2014a). In addition to the parent peptide, several derived fragments (LPLP and HLPL) were detected after both, intravenous and oral administration of the penta-peptide HLPLP. However, whether the derived fragments released in plasma could retain any biological activity remained to be demonstrated.

The aim of this study is to determine if any of the fragments that had been previously demonstrated to be released in vivo from the penta-peptide
HLPLP retain antihypertensive activity. To reach this objective, in a preliminary experiment, the fragments generated during incubation of the peptide in rat blood plasma were quantitatively analyzed by ultra-high performance liquid chromatography (UPLC) coupled on line to a quadrupole-time-of-flight (Q-TOF) analyzer. Identified fragments were chemically synthesized and their antihypertensive effect was evaluated in spontaneously hypertensive rats (SHR) after single oral administration. In addition, their ability to inhibit ACE was assessed in isolated aorta rings.

2. Materials and methods

2.1 Peptide synthesis and standard solution preparation.

Peptides were synthesized in-house by conventional fluorenyl-methoxy-carbonyl chloride (fmoc) solid-phase method, using a 431A peptide synthesizer (Applied Biosystems Inc. Überlingen, Germany). The synthesized peptides corresponded to β-casein sequences: HLPLP f(134-138), HLPL, f(134-137), HLP, f(134-136), LPLP, f(135-138), and LPL, f(135-137). Peptide PLP, β-casein f(136-138), was purchased from SynPeptide CO, LDT (Shanghai, China). Their purities were above 93%, calculated by RP-HPLC-UV-MS. All other chemicals were of the highest quality grade. The standard curves of HLPLP, HLPL, LPLP and HLP in plasma where prepared as previously described (Sánchez-Rivera et al., 2014a). Briefly, each synthetic peptide diluted in Milli-Q water was added to plasma to reach final peptide concentrations from 2.5 ng mL\(^{-1}\) to 20 µg mL\(^{-1}\). Immediately after, 40 µL of trifluoroacetic acid (TFA) solution (10%, v/v) were added, followed by heat treatment up to 99°C for 2 min. After centrifugation
(8500 × g, 30 min), the supernatant was purified using mixed mode action exchange cartridges (Bond Elut Plexa PCX, 60 mg, 1 ml, Agilent, Santa Clara).

2.2 Incubation of peptide β-casein f(134-138) in rat plasma.

The peptide HLPLP, β-casein f(134-138) was incubated in rat control plasma obtained from female Wistar rats (250-300 g). The rats were anaesthetized using pentobarbital at intraperitoneal dose of 60 mg kg⁻¹ body weight and the blood was extracted by cardiac puncture (5 mL per rat), with 5 mL 23G syringe. The blood was introduced in a lithium-heparin 9 mL tube, and centrifuged at 3600 rpm for 20 min at 4ºC. The supernatant was collected and frozen at -20ºC until use. Plasma was mixed 5:1 with the peptide aqueous solution to reach a final concentration of 0.06 mg mL⁻¹ (0.10 mM). This mixture was kept at 37ºC during the experiment in a thermally controlled water bath (AQUAline AL25, LAUDA WOBSE, GmbH & Co., Postfach, Germany). Sampling was done at 5, 30, 60, 120 and 240 min after peptide addition to the plasma, and also an aliquot was taken just after the peptide was spiked in plasma and vortexed for 10 seconds (T0). The incubation was stopped using TFA (10%, v/v) and the plasma samples were treated as described above.

2.3 UPLC-Q-TOF analyses.

The analyses of the plasma samples were performed on an UPLC from Waters (Milford, MA, USA), coupled to a MicroTOF-QII instrument (Bruker Daltonik, Germany). These analyses were carried out as described by Sánchez-Rivera et al. (2014a) with some modifications. The pseudo-selected ion monitoring (SRM) method was set up to fragment the following parent ions, with mass-to-charge ratio (m/z) of 576.34, 439.28, 479.29, 366.21, 342.23, and
326.20, which corresponded to the sequences HLPLP, LPLP, HLPL, HLP, LPL and PLP, respectively. The amount of peptide fragments at different incubation times was quantified by the extraction of their corresponding molecular ions (m/z) and expressed as concentration (μM) by the use of a standard curve for each peptide.

2.4 Pharmacological activity assays.

All the animal trials were carried out according to the European Union guidelines for the ethical care and use of laboratory animals (European Directive 2010/63/EU). 15 to 17-week old spontaneously hypertensive rats (SHR) were purchased from Elevage Janvier (Le Genest, Saint Isle, France). The animals were housed in groups of three rats and kept at 23°C with 12 h light/dark cycles in the Animal Experimentation Service of Salamanca University (NPAE SA001). Standard food (Global Diet 2014, Harlan, France) and water were available ad libitum.

2.4.1 Antihypertensive activity assays in SHR.

To determine the antihypertensive activity, the synthesized peptides dissolved in ultrapure water were orally administered using an oesophageal cannula, at a single dose of 7 mg kg\(^{-1}\) body weight, and the control group received the same volume of water. The animals were deprived of solid food diet 12 h before experiments, only having access to sucrose (80 g L\(^{-1}\)) and NaCl (2 g L\(^{-1}\)) solution. The systolic blood pressure (SBP) was measured as previously described (Sánchez et al., 2011), using the CODA tail-cuff blood pressure system (Kent Scientific, Torrington, CT, USA). The measurement of SBP was performed before peptide administration to estimate the basal blood
pressure, which was 155 mmHg for SBP, and after administration thereof at 2, 4, 6, 8 and 24 h. The changes in the measurement of SBP were expressed as mmHg.

2.4.2 ACE-inhibition assays in aorta rings.

To determine ACE inhibition, isolated thoracic aorta rings were mounted in organ bath with Krebs solution at 37°C aerated with carbogen as described elsewhere (Sánchez et al., 2011). After an equilibration period for 1 h at a resting tension of 2 g, the functionality of the rings was tested with a 120 mM solution of KCl. After that, rings were incubated with different peptides (HLPLP, LPLP, HLPL or HLP) at a dose $10^{-4}$ M (100 μM), for 30 min, and the contractile response to Ang I ($10^{-7}$ M) was evaluated. Captopril ($10^{-6}$ M) was used as positive control.

2.5 Statistical analysis.

The mean values are expressed with the standard error of mean (SEM). The effect of each administered peptide over time and versus the control (water) was recorded. Two-way analysis of variance (ANOVA), using GraphPad Prism 5.0 (GraphPad software Inc., San Diego, USA) was performed. Bonferroni post-test was applied to establish the significant differences between the peptides and the control. Contractile responses to Ang I were expressed as mg of contraction and statistical analysis was made using one way ANOVA comparing responses to Ang I in absence and presence of the peptides during the incubation period. Differences were considered significant at $P \leq 0.05$. 

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3. Results

3.1 Hydrolysis of β-casein f(133-138), HLPLP, by plasma peptidases.

To evaluate the resistance of β-casein f(133-138) HLPLP to plasma peptidases over time and to identify the formation of derived fragments, the penta-peptide was incubated in rat plasma for 4 h. Peptide was dissolved in plasma at a concentration of 0.06 mg mL\(^{-1}\) selected on the basis of previous in vivo experiments where the peptide was intravenously administered to rats (Sánchez-Rivera et al., 2014a). Blank plasma was previously analyzed to confirm that neither HLPLP nor derived fragments were naturally present in plasma before these experiments (data not shown). A disappearance of 20% for HLPLP was observed from time zero to 5 min (Figure 1a); although from this point to 30 min, 6-fold decrease was observed. Then, plasma peptidases caused a pronounced depletion on its concentration from 30 to 60 min of incubation (10-fold decrease, from 0.9 to 0.09 μM). Nevertheless, the peptide was detected until 240 min. The formation of all possible tetra- and tri-peptides was monitored by pseudo-SRM and three derived fragments were identified and quantified during incubation in plasma, i.e., LPLP, HLPL and HLP. The release of the peptide fragments occurred rapidly and the two tetra-peptides, HLPL (Figure 1b) and LPLP (Figure 1c) were detected in the first sample withdrawn from the incubation (time zero). These tetra-peptides suffered a decrease from 5 to 30 min, to 7.05% and 1.61%, respectively, of the initial amount detected in plasma. The first one, resisted during 60 min of incubation, however the latter one, was observed up to 120 min. The tri-peptide HLP (Figure 1d) was also detected at time zero, and increased significantly during the first 5 min of incubation in plasma, keeping a rising trend until 60 min; to finally suffer a
pronounced decrease, although it was still detected at 240 min of incubation. The concentration of HLPLP quantified at time zero was only 5.8% of the total amount initially added to plasma. At this point, its derived fragments LPLP, HLPL and HLP represented 24%, 0.5% and 7.8%, respectively, of the initial amount of HLPLP spiked into the plasma. These results sum up to 38.1% of the reference amount of HLPLP added to plasma. Thus, other peptide fragments, such as, LPL and PLP, and free amino acids might be released by the action of plasma peptidases, although the limitation of the MS-based technique to identify short peptides in plasma may have precluded their detection.

3.2 Evaluation of the antihypertensive activity in SHR.

In order to assess the antihypertensive activity of the fragments released from HLPLP, the parent peptide, and all possible derived tetra- and tri-peptides were chemically synthesized. Figure 2 shows the antihypertensive effect of the different β-casein peptides, orally administered to SHR at 7 mg kg⁻¹ body weight. This dose had been previously assayed for peptide HLPLP in SHR and produced a significant SBP decrease (Miguel, Gómez-Ruiz, Recio, & Aleixandre, 2010). The administration of HLPLP and its derived peptide fragments led to different behaviours in regard to the trend of SBP decrease. The parent peptide, HLPLP, (Figure 2a), showed a maximum decrease of the SBP at 2 h post-administration (21.1 ± 3.4 mmHg) and subsequent recovery occurred from this point. However, the two tetra-peptides, HLPL and LPLP (Figures 2b and 2c, respectively) caused a reduction on SBP that reached its maximum 4 h after administration (19.4 ± 3.8, and 16.2 ± 3.8 mmHg, respectively). The administration of the tri-peptides HLP, LPL and PLP (Figures 2d, 2e and 2f, respectively) to SHR led to a delay on the decrease of SBP
compared to the previous ones. HLP and LPL showed a maximum, peak of SBP reduction 6h after administration (15.2 ± 1.4 and 22.0 ± 2.5 mmHg, respectively). Finally, the administration of PLP induced the most effective SBP decrease in terms of endurance. This peptide caused a gradual drop of SBP values that reached its maximum at 8 h post-administration (21.2 ± 3.4 mmHg). The long-lasting effect of this peptide, led to a slow rate of recovery. At 24 h, significant differences with the control on the reduction of SBP were still observed.

3.3 ACE inhibition in aortic rings.

The response of angiotensin I in isolated aortic rings was assessed after incubation with the precursor peptide and the derived-fragments identified in plasma. Angiotensin-I is converted to Angiotensin-II by tissue ACE activity, resulting in a contractile response. Figure 3 shows that the contractile response to Angiotensin-I (780 +/- 100 mg) was significantly reduced in the presence of the peptides tested at 10^-4 M. The highest reduction (44% of the control) was achieved with HLPLP, but no significant differences were observed among all tested peptides. However, the reduction induced by the ACE inhibitor captopril (82% of the control, at 10^-6 M) was more efficient than that observed for HLPLP.

4. Discussion

Most studies on food-derived antihypertensive peptides associate the orally administered sequence with a given effect, although it is known that peptides might suffer hydrolysis during digestion, absorption and, if they reach circulation, further degradation by plasmatic peptidases. The region from β-casein 130-140 has been shown to be especially resistant to digestion and
peptides comprised in this region have been found by us and others after simulated gastrointestinal digestion of dairy products (Robert, Razaname, Mutter & Juillerat, 2004; Dupont et al., 2010; Picariello et al., 2013; Sánchez-Rivera et al., 2014b) and in human jejunal aspirates (Boutrou et al., 2013). The evidence of the absorption of the antihypertensive penta-peptide HLPLP, f(134-138), that can be formed after milk digestion represents a step forward in the study of bioavailability of food derived peptides. The kinetic parameters of absorption and elimination have revealed that HLPLP is rapidly absorbed and the oral bioavailability has been calculated on the basis of HLPLP concentration in plasma. Interestingly, it was observed that this penta-peptide was further hydrolyzed into smaller fragments in vivo, and the kinetic parameters for the biotransformed derived fragments, LPLP and HLPL, were also calculated, showing elimination half-life values of 8.38 and 10.9 min, respectively (Sánchez-Rivera et al., 2014a), although the formation of other biotransformed fragments could not be excluded. The route of the formation of these derived fragments and if they could exert antihypertensive activity remained to be ascertained.

In this work, the plasma incubation permitted to assure that the penta-peptide is degraded by the action of soluble blood plasma peptidases giving rise to, at least, two tetra-peptides and one tri-peptide, LPLP, HLPL and HLP. However, other peptide fragments were probably formed, known the limitation of MS for short peptides and as revealed by the mass balance. Two of the peptide fragments (LPLP and HLPL) identified in this study during in vitro incubation of the penta-peptide were coincident with those found in vivo after oral and intravenous administration of HLPLP in rats (Sánchez-Rivera et al.,
Thus, one of the routes of degradation of HLPLP *in vivo* probably involved plasma peptidases once the peptide reached blood circulation. The initial concentration of HLPLP detected in plasma after incubation was under the expected value, due to its fast degradation into its derived fragments. The increase in concentration undergone by HLP during incubation in plasma from 5 to 60 min coincides with the decrease monitored for HLPL and HLPLP. These results could indicate the formation of the tri-peptide from both the penta- and tetra-peptide, by the action of plasma peptidases. Moreover, the concentration calculated for HLPL is lower than that of LPLP and HLP, suggesting that HLPL could be further hydrolyzed into HLP. The concentrations of LPLP and HLP were higher than that of the precursor peptide (6 and 5-fold, respectively), showing the rapid and extensive hydrolysis of the penta-peptide into smaller peptide fragments even a few seconds after incubation (time zero). Similar results for the peptide fragment LPLP, were reported *in vivo* after administration of HLPLP to rats, where this tetra-peptide was found in plasma at higher concentration compared to its precursor (Sánchez-Rivera et al., 2014a). This immediate degradation of HLPLP is consistent with that observed for Angiotensin II in plasma, which occurred within seconds (Moskiwitz, 2003), and also with the rapid degradation reported for other dairy peptides such as, Lactokinin [β-lactoglobulin f(142-148)] in human serum (Walsh et al., 2004).

In view of the results, the main circulating peptide in blood after HLPLP ingestion could be LPLP, which has shown a significant antihypertensive effect, although less acute than the observed in the pentapeptide. For antihypertensive peptides where absorption has been demonstrated, the required plasma concentration known to exert the biological effect was approximately 1000-fold.
higher than the plasma concentrations reported. This was the case of the casein-derived tripeptide IPP in a clinical study involving ingestion of a lactotripeptide enriched yogurt beverage. Maximal plasma concentrations were in the picomolar level and an elimination half-life of 30 min was determined (Folz, Meynen, Bianco, van Platerink, Koning, & Kloek., 2007). In a pharmacokinetic study in piglets, where intragastric administration of synthetic IPP, LPP and VPP was conducted, the calculated bioavailability was 0.1% and the determined elimination half-lives ranged from 5 to 20 min (van der Pijl, Kies, Ten Have, Duchateau, & Deutz, 2008). The present work supports that plasma concentration of peptides might be underestimated due to biotransformation of the peptide into other active fragments. In contrast, for the dipeptide VY ingested in an enriched beverage, plasma concentrations in the nanomolar level were reached but a long half-life of 3.1 h was observed (Matsui, Tamaya, Seki, Osajima, Matsumoto, & Kawasaki, 2002). These values suggest an impaired degradation of the peptide.

With regard to the observed effects for the different peptides, it has to be pointed out that many biologically active peptides are protected from general proteolytic degradation by evolutionary conserved prolines. Interestingly, the PLP sequence was one of the common motifs employed by Juillerat-Jeanneret, Robert, & Juillerat (2011) to identify inhibitors of prolyl-specific proteases comprised in the αs1- and β-casein sequences. The high antihypertensive potency shown by peptide PLP is consistent with its structure, which is favorable to bind and inhibit ACE (Quirós et al., 2009). In addition, it could inhibit prolyl-proteases activity which might explain the endurance of the effect exerted by this tri-peptide.
In view of the results obtained on aortic rings, it is proved that the antihypertensive effect of peptide HLPLP and those produced by the action of blood plasma peptidases is due, at least in part, to its ability to inhibit ACE. These results are in agreement with previous studies where the peptide HLPLP showed antihypertensive activity and inhibited ACE activity *in vitro* (IC$_{50}$ 21 μM) (Hernández-Ledesma, Quirós, Amigo, & Recio, 2007). However, the effect found for and LPLP on aortic rings at 100 μM was higher than that expected based on the reported *in vitro* ACE-inhibitory activity (IC$_{50}$ 720 μM, respectively) (Kohmura, Nio, Kubo, Minoshima, Munekata, & Ariyoshi, 1989). Similarly, IPP and VPP have been reported as ACE inhibitors *in vitro* with an IC$_{50}$ value at micromolar level (Nakamura, Yamamoto, Sakai & Takano, 1995). In addition, these tri-peptides have also shown ACE-inhibitory activity in mesenteric artery rings after 24h of incubation with the tri-peptides (Jäkälä, Jauhiainen, Korpela & Vapaatalo, 2009).

5. Conclusions

The incubation of the antihypertensive peptide β-casein f(134-138) HLPLP in rat blood plasma permitted the identification of novel derived fragments (HLPL, LPLP and HLP), which may be physiologically relevant in respect of the antihypertensive effect. These peptide fragments are coincident with those previously found *in vivo*. Some of the fragments found in this study, such f(134-136) HLP, were detected until 240 min of incubation time. All possible peptide-fragments derived from HLPLP retained antihypertensive activity when orally administered to SHR. Our results after oral administration
have allowed the identification of new active β-casein tri-peptides, which could be produced by fermentation or enzymatic hydrolysis as enrichment strategies. From all the peptide fragments, the tri-peptide PLP showed the longest lasting antihypertensive effect which was significant up to 24 h after administration. The incubation of HLPLP in plasma, which was known to be absorbed intact into circulation, was a good *in vitro* approach to follow its further degradation by plasmatic peptidases, since the identified fragments were in accordance to those found *in vivo* after administration of HLPLP to rats. These results should be taken into account in the overall physiological effect of the penta-peptide HLPLP. Inhibition of ACE is involved in the mechanism of antihypertensive activity of these peptides, although further studies should be conducted to explore if other possible mechanisms could be concerned.

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References


Figure Captions

**Figure 1.** Concentrations (µM) of the peptides HLPLP (a), HLPL (b), LPLP (c) and HLP (d) identified during the incubation of the penta-peptide in plasma. Mean values ± SEM (n=3).

**Figure 2.** Systolic blood pressure decrease (Δ) caused in spontaneously hypertensive rats after administration of water (*), or 7 mg kg⁻¹ of body weight of HLPLP (a), HLPL (b), LPLP (c), HLP (d), LPL (e) and PLP (f). Mean values ± SEM (n=5-6) are represented. Statistical differences were found at p≤0.05 (*), p≤0.01 (**) and p≤ 0.001(***).

**Figure 3.** Responses to Angiotensin I (Ang I, 10⁻⁷ M) in aortic rings in absence (control) and presence of the peptides (10⁻⁴ M) or captopril (10⁻⁶ M) after the incubation for 30 min. Mean values ± SEM (n=5-10) are represented. * p≤0.05, *** p≤0.001 versus control.
Figure 1
Figure 3

[Bar graph showing contractile response to Ang (mg) for different groups: Control, HLPLP, HLPL, LPLP, HLP, and Captopril. The graph indicates significant differences (*) among the groups.]
Highlights

Fragments generated by plasmatic peptidases on peptide HLPLP have been identified.

Three peptides coincide with those found in vivo after HLPLP administration to rats.

All possible derived peptides from HLPLP show potent antihypertensive activity.

The peptides caused inhibition of vascular contraction elicited by angiotensin-I.