Resistance of casein-derived bioactive peptides to simulated gastrointestinal digestion

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Abbreviations: ACE, angiotensin-converting enzyme; IC_{50}, concentration necessary to inhibit 50% of ACE activity; ORAC-FL, oxygen radical absorbance capacity-fluorescein; SBP, systolic blood pressure; SHR, spontaneously hypertensive rats.

Abstratct

The resistance of six casein-derived peptides, including antihypertensive peptides RYLGY, AYFYPEL and YQKFPQY, to simulated gastrointestinal digestion and the effect on activity were evaluated. After digestion, peptides RYLGY, AYFYPEL, and YQKFPQY were partly hydrolyzed by digestive enzymes. From these, RYLGY and AYFYPEL maintained a potent ACE-inhibitory activity, with IC_{50} values as low as 9.3 and 4.7 $\mu g\ mL^{-1}$, respectively. Several of the digestion fragments, previously identified by HPLC-MS and synthetized, showed potent ACE-inhibitory, which could explain the *in vitro* activity of the digests. A notable antioxidant activity was also observed. Furthermore, since AYFYPEL was less susceptible to digestion, we focused on the antihypertensive activity in SHR of the main digestion fragments of RYLGY. Interestingly, these peptides showed moderate effects *in vivo*. This finding suggests that, besides undigested fraction that could also contribute in the *in vivo* effects of RYLGY and AYFYPEL, other minor fragments may participate.

1. Introduction

It is known that bioactive peptides may undergo physiological transformations that determine their bioavailability and activity in the organism. In fact, gastrointestinal digestion play an important role in the formation and degradation of bioactive peptides. Although *in vivo* methods, using animals or humans, usually provide the most accurate results, they are costly and time consuming. Therefore, a great interest has been dedicated on the development and application of *in vitro* approaches (Hur, Lim, Decker, & McClements, 2011). Sequential hydrolysis with pepsin and pancreatic extracts have been applied to mimic the gastrointestinal conditions and to evaluate the stability of angiotensin-converting enzyme (ACE) inhibitory peptides. This hydrolysis process together with *in vitro* studies with epithelial intestinal Caco-2 cells have allowed elucidating whether the released sequences have physiological relevance in blood pressure regulation. For instance, casein-derived peptides IPP and VPP, have been demonstrated to be highly resistant to digestive peptidases (Ohsawa et al., 2008), to be absorbed through intestinal epithelium (Foltz et al., 2008), reaching blood circulation in an intact and active form (Van Platerink, Janssen, Horsten, & Haverkamp, 2006; Foltz et al., 2007). Following a similar procedure, it was found that peptide LHLPLP resisted simulated gastrointestinal digestion, but it was hydrolysed by intestinal peptidases to the active form, HLPLP, prior to its absorption (Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008; Quirós, Contreras, Ramos, Amigo, & Recio 2009). Interestingly, this pentapeptide was detected in human plasma samples after the consumption of a peptide-enriched drink (Van Platerink et al., 2006).

Gastrointestinal digestion may also lead the formation of active fragments from inactive or less active precursors. For example, the antihypertensive β-CN fragment, KVLPVP, was released from the inactive precursor KVLPVPQ after pancreatic digestion (Maeno, Yamamoto, & Takano, 1996). Miguel, Aleixandre, Ramos, & López-Fandiño (2006)

found, after simulated gastrointestinal digestion of egg white-derived peptides YAEERYPIL and RADHPFL, that released fragments YPI and RADHP might contribute on the *in vivo* effects of precursor peptides.

Two potent ACE-inhibitory peptides were identified in a peptic casein hydrolysate which corresponded to RYLGY [α_{S1}-CN f(90–94)], and AYFYPEL [α_{S1}-CN f(143–149)] (Contreras, Carrón, Montero, Ramos, & Recio, 2009). These peptides also exerted significant antihypertensive activity in spontaneously hypertensive rats (SHR) after both oral acute and long-term administration (Contreras et al., 2009; Sánchez et al., 2011). Additional cardiovascular benefits, such as significant improvement of the aorta and mesenteric acetylcholine relaxations, increase of the aortic endothelial nitric-oxide synthase (eNOS) expression and decrease of the left ventricular hypertrophy and intersticial fibrosis were also observed in SHR after administration of this casein hydrolysate (Sánchez et al., 2011). The hydrolysis process was scaled up to produce, under food grade conditions, an antihypertensive ingredient containing the most active peptides RYLGY and AYFYPEL (Contreras et al., 2011). Furthermore, no treatment-related toxicity was detected when casein hydrolysate was orally administered to Wistar rats at doses of 1000 mg kg^{-1} for 4 weeks or 2000 mg kg^{-1} in a single oral dose, which supports its safe use as functional ingredient (Anadón et al., 2010).

The main objective of the present study was to evaluate the stability of peptides previously identified in a casein hydrolysate against a process simulating physiological gastrointestinal digestion. Moreover, ACE-inhibitory, antihypertensive, and antioxidant activities of peptide fragments released during digestive process were also investigated in order to identify the *in vivo* active sequences.

2. Material and methods

2.1. Peptide synthesis

All peptides were prepared by conventional Fmoc solid-phase synthesis, using a 431A peptide synthesizer (Applied Biosystems, Uberlingen, Germany). The purity of these 16 peptides was verified by analytical RP-HPLC-MS (Quirós et al., 2008). Peptides corresponded to six casein-derived sequences found in a peptic hydrolysate: RYLGY, AYFYPEL, FVAPFPEV, VAPFPEVF, YQKFPQY, and HLPLPLL; and peptide fragments there of generated during simulated gastrointestinal digestion: RY, YLG, RYLG, LGY, RYL, YLGY, AYFYPE, FYPEL, YQK, and FPQY were studied.

2.2. Simulated gastrointestinal digestion

The two stage-hydrolysis process simulating gastrointestinal digestion was carried out according to Gómez-Ruiz, Ramos, & Recio (2004) with the exception that the enzyme employed was pepsin (E.C. 3.4.23.1; 1:10,000; 570 U mg^{-1}) (Sigma, St. Louis, MO, USA). The simulated gastrointestinal digestion was carried independently and in duplicate

2.3. Peptide sequencing by RP-HPLC-MS/MS

Digests were analysed by RP-HPLC-MS/MS using an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) connected on-line to an Esquire 3000 quadrupole ion trap (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ionisation source, as previously described (Contreras et al., 2010).

2.4. Measurement of ACE-inhibitory activity

ACE-inhibitory activity was measured by spectrophotometric assay of Cushman & Cheung (1971) with some modifications, as reported by Quirós et al. (2007). The ACE-inhibitory activity of the samples was expressed as IC_{50} (peptide concentration required to inhibit the original ACE activity by 50%) and was determined in triplicate.

2.5. Measurement of antioxidant activity

Oxygen radical absorbance capacity-fluorescein (ORAC-FL) assay was based on the method applied by Dávalos, Gómez-Cordovés, & Bartolomé (2004) and reported by Contreras, Hernández-Ledesma, Amigo, Martín-Álvarez, Recio (2011). This assay was also performed in triplicate for each sample.

2.6. Measurement of antihypertensive activity

All the experiments were performed according to the European Union guidelines for the ethical care and use of laboratory animals (European Directive 86/609/CEE). Male SHR 12 week old (Elevage Janvier, Le Genest Saint Isle, France) were housed in groups of four rats and maintained at 23ºC with 12 h light/dark cycles. The rats received, by oral administration using a canula, a single dose of the synthesized peptides (5 mg kg^{-1} of body weight) dissolved in ultrapure water. Systolic blood pressure (SBP) was measured in awake rats using the CODA tail-cuff blood pressure system (Kent Scientific, Torrington, CT, USA) as was described previously (Sánchez et al., 2011). Blood pressure measurement was carried out before peptides administration, and at different times post-administration.

2.7. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical calculations for significant differences between SBP before administration and the different times postadministration were performed by Student's t test for paired data and P values of less than 0.05 was considered significant. The GraphPad Prism 5 software program (GraphPad Software Inc., San Diego, California, USA) was used.

3. Results and discussion

3.1. Effect of simulated gastrointestinal digestion on ACE-inhibitory activity of casein peptides

The hydrolysis pattern of synthetic casein peptides subjected to simulated gastrointestinal digestion was evaluated by HPLC-MS/MS. This technique also allowed the identification of peptide sequences released during the digestive process. As shown in Fig. 1, six peptides were hydrolysed by digestive enzymes, but the degree of hydrolysis was different. Peptides RYLGY, YQKFPQY, AYFYPEL, and FVAPFPEV were partially resistant to gastrointestinal digestion, whereas peptides VAPFPEVF and HLPLPLL were totally hydrolysed. After the action of digestive enzymes, 93% of peptide RYLGY was hydrolysed, and RY, YLG, RYLG, YLGY, and RYL (Fig. 1A) appeared as major peptide fragments in the final digest. In the case of peptide AYFYPEL, 47% of undigested peptide remained intact at the end of the digestive process (Fig. 1B). This peptide and a shorter peptide, YFYPEL, were also found after simulated gastrointestinal digestion of different milk

products, such as infant formula (Hernández-Ledesma, Quirós, Amigo, & Recio, 2007), milk and yoghurt (Dupont et al., 2010). Moreover, a study performed in humans consuming milk or yoghurt demonstrated the presence of peptides AYFYPEL and YFYPEL in the stomach and duodenum, respectively, which demostrate the resistance of this α_{s1}-casein fragment to human digestion (Chabance et al., 1998).

Several studies have suggested the role of amino acid proline making peptides less susceptible to proteolytic action (Quirós et al., 2007; Ohsawa et al., 2008), although the effect is dependent on the amino acid's position (Gómez-Ruiz et al., 2004). The presence of two inner prolines conferred resistance to the action of endopeptidases to peptides FVAPFPEV and VAPFPEVF, but their susceptibility to aminopeptidases and mainly carboxypeptidases was different. Peptide FVAPFPEV was weakly hydrolysed into VAPFPEV and FVAPFPE (Fig. 1C), whereas VAPFPEVF was completely hydrolysed, releasing the peptide VAPFPEV (Fig. 1D). In the case of YQKFPQY, trypsin could hydrolyse the peptide bond between lysine and phenylalanine, releasing the major fragments YQK and FPQY that were detected in the digest (Fig. 1E). HLPLPLL was also totally hydrolysed by digestive enzymes, releasing HLPLPL and a minor fragment that corresponded to the active form, HLPLP (Fig. 1F).

ACE-inhibitory activity of casein peptides, before and after digestion, is shown in Fig. 2. The most active sequences were RYLGY, AYFYPEL, YQKFPQY, and HLPLPLL, with IC_{50} values of 0.5, 5.9, 19.5 and 27.6 μg mL^{-1}, respectively. However, peptides FVAPFPEV and VAPFPEVF showed low inhibitory activity with IC_{50} values higher than 300 μg mL^{-1}. The action of digestive enzymes increased the activity of peptides AYFYEPL and FVAPFPEV, disminishing the activity of the rest of analysed peptides. It is noteworthy to mention the potent ACE-inhibitory activity measured in digests obtained from peptides RYLGY (IC_{50} = 9.3 μg mL^{-1}), and AYFYPEL (IC_{50} = 4.7 μg mL^{-1}). This activity was notably higher than that reported for other peptides obtained under similar conditions and exhibiting

antihypertensive activity *in vivo* (Quirós, Hernández-Ledesma, Ramos, Amigo, & Recio, 2005; Miguel et al., 2006; Hernández-Ledesma, Miguel, Amigo, Aleixandre, & Recio, 2007).

3.2. ACE-inhibitory and antioxidant activities of fragments released from casein peptides

The main fragments released from peptides RYLGY, AYFYPEL and YQKFPQY, after simulated gastrointestinal digestion, were chemically synthesized and their ACE-inhibitory and radical scavenging activities were evaluated (Table 1). After digestion of potent ACE-inhibitory peptide RYLGY, six sequences were identified, with three of them; LGY, YLGY, and RY showing IC_{50} values lower than 55 μM. These sequences and the remaining precursor peptide could contribute to the activity detected in the final digest. Presence of Tyr at C-terminal position could favour the peptide binding to ACE (Igarashi et al., 2006; Tavares et al., 2011). The major peptides released from AYFYPEL, whose sequence were AYFYPE and FYPEL, showed moderate activity with IC_{50} values of 260.82 μM and 80.60 μM, respectively. This result indicates that intact precursor peptide could be the sequence responsible for the high activity detected in the hydrolysate obtained at the end of simulated gastrointestinal digestion. Moreover, minor peptides could also contribute to this activity. Yang, Tao, Liu, & Liu (2007) found that dipeptide AY was a true inhibitor of ACE, with an IC_{50} value of 14.2 μM.

In the case of sequence YQKFPQY, it was totally hydrolysed and major released fragments, YQK and FPQY showed higher IC_{50} values (312.23 μM and 300.74 μM, respectively) than that of the precursor peptide (20.08 μM), thus explaining the decrease of the activity detected in the final digest.

Precursor casein peptides (RYLGY, AYFYPEL, and YQKFPQY), and their derived fragments after action of digestive enzymes showed ORAC-FL values higher than that of

212 Trolox, used as standard antioxidant. These values were ranged from 1.38 to 4.16 μmol of
213 Trolox μmol^{-1} of peptide (Table 1). Presence of Tyr in all these peptides could determinate the
214 high antioxidant activity detected. This amino acid had been previously described as
215 responsible for radical scavenging activity of food-derived peptides (Dávalos, Miguel,
216 Bartolomé, & López-Fandiño, 2004; Hernández-Ledesma, Dávalos, Bartolomé, & Amigo,
217 2005; Hernández-Ledesma, Amigo, Recio, & Bartolomé, 2007). Moreover, those peptides
218 containing two residues of Tyr were the most potent sequences identified in this study. In the
219 case of peptides RYLGY and YQKFPQY, precursor sequences were more active than the
220 released peptides, which could be due to the presence of two Tyr within the sequence, one of
221 them being a C-terminal amino acid. However, peptide AYFYPE released during digestion
222 process showed an ORAC-FL value of 4.16 μmol of Trolox μmol^{-1} of peptide, 1.3-times
223 higher than that shown by its precursor peptide AYFYPEL. Deletion of Leu at C-terminal
224 position enhanced antioxidant activity, leaves Tyr at antepenultimate position and it seems to
225 improve radical scavenging.
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227 *3.3. Antihypertensive activity of casein-peptides derived fragments*
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229 Since peptide RYLGY showed potent antihypertensive activity but low resistance to
230 enzymatic digestion, this peptide was selected to investigate its antihypertensive activity of
231 the major released fragments, i.e., RYLG, YLGY, RY, and YLG, after gastrointestinal
232 digestion. Fig. 3 shows the changes in systolic blood pressure (SBP) of SHR after acute
233 administration of 5 mg peptide kg^{-1} of body weight. The precursor peptide, RYLGY, notably
234 reduced SBP after 4 post-administration, obtaining decreases around 20 mm Hg. All peptide
235 fragments, except YLG, exerted significant hypotensive activity and no statistically
236 significant differences were found among peptides (except for YLG). Among the released

237 fragments, RYLG exhibited significant decreases ($p<0.05$) at 2, 4 and 8 h post-
238 administration; RY at 4 and 8 h post-administration and YLGY at 2, 4, 6 and 8 h post-
239 administration. Three of the peptides assayed have Tyr as C-terminal residue as other food
240 derived peptides that have demonstrated to be absorbed and reach blood circulation in an
241 intact form and to exert hypotensive effects in SHR (Matsufuji et al., 1995; Matsui et al.,
242 2002; Van Platerink et al., 2006; Foltz, et al., 2007). The antihypertensive activity on SHR of
243 peptide RY had been previously demonstrated and its effects continued for 30 h after oral
244 administration (Saito, Wanezaki, & Imayasu, 1994).

245 Peptide RYLG reached the maximum antihypertensive effect after 6 h post-
246 administration (-18. ± 5 mm Hg). However, ACE-inhibitory activity of this peptide was low,
247 with an IC_{50} value of 224.7 μM. This result opens up the possibility that, besides ACE-
248 inhibitory activity, other molecular mechanisms underlie the mode of action of this peptide.
249 Increased production of superoxide anion and hydrogen peroxide, reduced nitric oxide
250 synthesis, and decreased bioavailability of antioxidants have been related with hypertension
251 (Touyz, 2004; Sánchez et al., 2011). Supplementation with antioxidants have been shown to
252 decrease blood pressure in animal models and humans with essential hypertension (Vasdev, &
253 Gill, 2005). Peptide RYLG showed a notable radical scavenging activity, indicating that its
254 antioxidant properties might contribute to its antihypertensive effect. Moreover, this peptide
255 presents high homology with sequence RYLGYL, α_{S1}-CN f(90–95), which has previously
256 been described as an opioid (Loukas, Varoucha, Zioudrou, Streaty, & Klee, 1983).
257 Hypotensive and vasodilator effects exerted by opioid peptides, such as of α-La f(50-53)
258 (YGLF), could be mediated by interaction with opioid receptors (Nurminen et al., 2000).
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260
261

4. Conclusions

Antihypertensive peptides RYLGY and AYFYPEL partly resisted the action of enzymes during a process simulating gastrointestinal conditions. High ACE-inhibitory activity of these peptides was still observed after simulated gastrointestinal digestion, with IC_{50} values lower than 10 μM mL^{-1}. All studied peptide fragments exhibited radical-scavenging activity, being AYFYPE the most active peptide, and several of them also showed ACE-inhibitory activity, such as LGY, YLGY and RY. The antihypertensive activity in SHR of RYLGY peptide was as high as that found after oral administration of RYLG, YLGY, and RY, main digestion fragments. This result highlighted that in addition to the undigested fraction, other minor fragments and their combined action could contribute in the *in vivo* effects of RYLGY. Although ACE-inhibition could be responsible of the antihypertensive activity, other mechanisms such as vasodilatation, antioxidant and opioid activity cannot be discarded. Thus, further work will be needed to clarify the physiological relevance and mechanism of other minor digestion fragments and the molecular mechanism of action of RYLGY.

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407 *Chemistry, 55*, 7891-7895.

FIGURE CAPTIONS

Figure 1. RP-HPLC chromatograms and peptide fragments released after simulated gastrointestinal digestion of synthetic petpides: (A) RYLGY; (B) AYFYPEL; (C) FVAPFPEV; (D) VAPFPEVF; (E) YQKFPQY and (F) HLPLPLL.

Figure 2. Angiotensin-converting enzyme-inhibitory activity, expressed as IC_{50} ($\mu g\ mL^{-1}$), of casein derived-peptides before ☐ and after ■ simulated gastrointestinal digestion. Data are expressed as mean ± standard deviation (n =3).

Figure 3. Effects of synthetic peptides RYLGY, RYLG, RY, YLGY, and YLG on systolic blood pressure (SBP) of spontaneously hypertensive rats (SHR) at different times after oral administration at the same dose. Data are expressed as mean ± SEM. Student's t test was used to compare different times post-administration (n = 6, *P < 0.05).