Novel casein-derived peptides with antihypertensive activity

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Abstract

In this study, we report novel casein-derived peptide sequences with angiotensin converting enzyme (ACE)-inhibitory activity and antihypertensive activity demonstrated in spontaneously hypertensive rats (SHR). The peptides were obtained by enzymatic hydrolysis of total isoelectric casein with pepsin. To identify ACE-inhibitory peptides, the casein hydrolysate was fractionated by semi-preparative high performance liquid chromatography, and 44 peptides contained in the active fractions were sequenced by using an ion trap mass spectrometer. Among the identified peptides, three sequences, that corresponded to α_{s1}-CN f(90-94) (RYLGY), α_{s1}-CN f(143-149) (AYFYPEL), and α_{S2}-CN f(89-95) (YQKFPQY), showed IC_{50} values as low as 0.71 μM, 6.58 μM, and 20.08 μM, respectively. These three peptides also exerted antihypertensive activity when they were orally administered to SHR at a dose of 5 mg kg^{-1} of body weight. The activity of peptides RYLGY and AYFYPEL in SHR was similar to that found for VPP when orally administered at the same dose.

1. Introduction

In recent years, extensive scientific evidence has been provided for the existence of biologically active peptides derived from food proteins that may have beneficial effects on human health. These protein fragments, known as bioactive peptides, can be released from the precursor inactive protein during gastrointestinal digestion and/or during food processing. Among the different groups of bioactive peptides, angiotensin converting enzyme (ACE)-inhibitory peptides are receiving special attention due to the prevalence and importance of hypertension in the Western population (for recent reviews see López-Fandiño, Otte, & van Camp, 2006; Murray & FitzGerald, 2007). Given the important role of diet in the prevention and treatment of hypertension, particular interest has been given to the production of foods with antihypertensive activity. It has now been demonstrated that the consumption of food products containing antihypertensive peptides produces a significant reduction in blood pressure (Jauhiainen & Korpela, 2007).

The release of the ACE-inhibitory peptides from milk proteins is commonly achieved by two different approaches: milk fermentation and milk protein hydrolysis. The first approach exploits the proteolytic system of lactic acid bacteria to hydrolyze milk caseins during the manufacture of dairy products, such as fermented milk and cheese (reviewed by Fitzgerald & Murray, 2006). The first fermented milk with antihypertensive activity was produced with a combination of *Lactobacillus helveticus* and *Saccharomyces cerevisiae* and contained two ACE-inhibitory tripeptides, VPP and IPP (Nakamura, Yamamoto, Sakai, & Takano, 1995). These two peptides have also been found in milk fermented with *Lactobacillus helveticus* LBK-16H. These peptides exhibited potent ACE-inhibitory activity and showed antihypertensive effects in both animals and humans (Sipola et al., 2001; Sipola, Finckenberg, Korpela, Vapaatalo, & Nurminen, 2002; Xu et al., 2008). Subsequently, our group, has shown the possibility of using different strains of *Enterococcus faecalis* to produce

fermented milk with potent ACE-inhibitory activity and antihypertensive activity in spontaneously hypertensive rats (SHR), after short- and long-term intake (Miguel et al., 2005; Muguerza et al., 2006). The peptide β-casein f(133-138), with sequence LHLPLP, has been identified as one of the major peptides responsible for the ACE-inhibitory and antihypertensive activities of the fermented milk produced (Quirós et al., 2007). Bioavailability studies of the antihypertensive peptide LHLPLP using Caco-2 cells have shown that LHLPLP is hydrolysed by cellular peptidases to HLPLP prior to the transport across the intestinal epithelium (Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008).

The second approach consists in the enzymatic hydrolysis of milk protein preparations by using one, or a combination, of food-grade enzymes to produce a large number of peptides, including peptides with antihypertensive effects. This strategy has been widely used to produce digests from caseinates, individual casein fractions, whey protein concentrates and isolates and individual whey proteins (reviewed by López-Fandiño et al., 2006; Murray & FitzGerald, 2007). Highly proteolytic microbial enzymes, such as proteinase K or thermolysin, have allowed the hydrolysis of whey proteins, which are more compact than caseins, to generate antihypertensive peptides (Abubakar, Saito, Kitazawa, Kawai, & Itoh, 1998; Hernández-Ledesma, Miguel, Amigo, Aleixandre, Recio, 2007b; Otte, Shalaby, Zacora, Pripp, El-Shabrawy, 2007b). However, the most potent antihypertensive and ACE-inhibitory-peptides are generated from caseinate or casein fractions (Otte et al., 2007b), probably related to the abundance of proline residues in the casein fractions and therefore, in the resulting peptides. Several proline-rich antihypertensive peptides have been identified from casein hydrolysates with a proteinase from *Lactobacillus helveticus*, with the sequences KVLPVP, KVLPVPQ, YKVPQL (Maeno, Yamamoto, & Takano, 1996), and several peptides with structures X-P and X-PP were produced with a protease from *Aspergillus oryzae* (Mizuno, Nishimura, Matsuura, Gotou, & Yamamoto, 2004). Digestive enzymes have also been employed for the release of antihypertensive peptides from the milk casein fraction, for

instance, trypsin (Karaki et al., 1990) or pepsin (López-Expósito, Quirós, Amigo, & Recio, 2007; Miguel, Contreras, Recio, & Aleixandre, 2009). Pepsin, has previously been employed to produce antihypertensive peptides from other substrates, such as, porcine skeletal muscle (Katayama et al., 2008), fish by-products (Je, Park, Kwon, & Kim, 2004) and egg proteins (Miguel, Recio, Gómez-Ruiz, Ramos, & López-Fandino, 2004). The ability of this enzyme to produce antihypertensive peptides is probably due to its preference for cleavage between hydrophobic residues.

Although there are numerous reports about the production of potential antihypertensive peptides from food proteins (of milk and non-dairy origin), their antihypertensive activity is not always evaluated and demonstrated in model animal systems, and most reports are based on a measurement of ACE-inhibitory activity. There are several examples of peptides, which showed potent ACE-inhibitory activity, but did not exert an antihypertensive effect in vivo. For instance, peptide α_{S1}-casein f(23-27) showed potent ACE-inhibitory activity, but no hypotensive effect in SHR (Maruyama et al., 1987), and peptide β-lactoglobulin f(142-148) with in vitro ACE-inhibitory activity but no effect in human volunteers (Walsh et al., 2004).

The objective of this study was to identify novel peptides with antihypertensive activity from an enzymatic hydrolysate of bovine casein. The strategy to identify novel peptide sequences was similar to that reported in previous study (Quirós et al., 2007), that is, fractionation of the hydrolysate, measurement of the ACE-inhibitory activity of each fraction, and sequencing of peptides contained in the active fractions by HPLC coupled to tandem mass spectrometry (MS/MS). Finally, the activity of chemically synthesized peptides was evaluated in vitro and in SHR, to ensure in vivo activity.

2. Materials and methods

2.1. Production of casein hydrolysates

A 0.5% (w/v) aqueous solution of isoelectric casein from raw skim milk, was adjusted to pH 2.0-2.5 with 1 M HCl and digested with 3.7% (w/w of substrate) porcine pepsin A (Sigma, St. Louis, MO, USA) for 24 h at 37 ºC in an orbital shaker at 150 rpm. The same amount of pepsin was added at the beginning and after 4 h of hydrolysis. Aliquots of the hydrolysate were taken during hydrolysis at 1, 2, 3, 4, 5, 6, 7 and 24 h. The reaction was terminated by heating at 80 ºC for 15 min and the pH was adjusted to 7.0 by addition of 1 M NaOH. The hydrolysates were centrifuged at 16,000 × *g* for 15 min and the supernatants collected. The protein concentration of supernatants was determined by the Kjeldahl method and ACE-inhibitory activity was tested.

2.1. Isolation of ACE-inhibitory peptides from the peptic hydrolysate

The 3-h peptic hydrolysate was subjected to ultrafiltration through a hydrophilic 3000 Da cut-off membrane (Millipore Corporation, Bedford, MA, USA) with a stirred ultrafiltration cell (model 8400, Millipore). The permeate from this ultrafiltration step was subjected to semi-preparative reverse phase high performance liquid chromatography (RP-HPLC) on a Waters Series 600 HPLC, equipped with Millennium 3.2 Software for data acquisition (Waters Corporation, Mildford, MA, USA) as previously described (Quirós et al., 2007). However, elution was performed with a linear gradient of solvent B in A from 0% to 21% B in 30 min, 21% to 35% in 40 min and to 35% to 70% in 5 min at 30 ºC and a flow rate of 4 mL min^{-1}. Solvent A was a mixture of water-trifluoroacetic acid (1000:1) and solvent B

contained a mixture of acetonitrile-trifluoroacetic acid (1000:0.8). Fractions from the HPLC system were freeze-dried and kept at –20 ºC until use.

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

RP-HPLC-MS/MS analysis of the active fractions was performed on an Agilent 1100 HPLC System (Agilent Technologies, Waldbron, Germany) with column HiPore® (RP318 C_{18} column 250 × 4.6 × 5 mm of particle size ???; Bio-Rad, Richmond, CA, USA). The HPLC system was connected on-line to an Esquire 3000 quadrupole ion trap (Bruker Daltonik GmbH, Bremen, Germany) equipped with an electrospray ionisation source, as previously described (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005).

2.4. Measurement of ACE-inhibitory activity

ACE-inhibitory activity was measured using the spectrophotometric assay of Cushman and Cheung (1971) with some modifications, as reported by (Quirós et al., 2007). For this purpose, protein concentration of the aliquots of the pepsin hydrolysate was determined by the Kjeldahl method. The protein content of the fractions collected from HPLC was estimated by the bicinchoninic acid assay (Pierce, Rockford, IL, USA) using bovine serum albumin, as standard, and peptide concentration of the chemically synthesized peptides was based on the dry weight of the peptides. Chemical synthesized peptides were purchased from GenScript Corp. (Piscataway, NJ, USA).

2.5. Measurement of antioxidant activity

160 The ORAC-FL assay was based on the method described by Ou, Hampsch-Woodill,
161 and Prior (2001) and modified by Dávalos, Gómez-Cordovés, & Bartolomé. (2004).
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163 *2.6. Measurement of blood pressure*
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165 All procedures were carried out in accordance with conventional guidelines for
166 experimentation with animals. Twelve-week old male SHR rats were used (Janvier, Le Genest
167 Saint Isle, 53940 France); they were housed in groups of four per cage in a regulated
168 environment with a 12 h light/dark cycle in a standard experimental laboratory of the Animal
169 Experimentation Service of the Salamanca University (NºPAE SA001). The animals were
170 deprived of solid diet 12 hours before experiments began but received a nutritive solution of
171 8% sucrose in 0.2% NaCl to avoid excessive dehydration. The rats received, by oral
172 administration, using a canula, a single dose of the synthesized peptides or zofenopril (5 mg
173 kg^{-1}) dissolved in ultrapure water. The control group received the same volume of water.
174 Systolic blood pressure (SBP) was measured in awake rats with an automated multichannel
175 system, using the tail-cuff method with a photoelectric sensor (Niprem 546, Cibertec SA,
176 Spain) as previously described (Guerrero et al., 2003). SBP was measured before
177 administration, and also at 2, 4, 6, 8, 10 and 24 h post-administration. The changes in SBP
178 were expressed as the differences before and after administration of the different products.
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180 *2.6. Statistical methods*
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182 Data are expressed as means +/- SEM. Statistical calculations for significant
183 differences between the different groups were performed by Student's t test for unpaired data
184 and P values of less than 0.05 were considered significant. The GraphPad Prism 4 software
185 program (GraphPad Software Inc., San Diego, California, USA) was used.
186

3. Results

3.1. Evolution of ACE-inhibitory activity during casein hydrolysis

The progress of the hydrolysis reaction of casein with pepsin was monitored by taking samples at different intervals. The water-soluble fractions of the hydrolysates were analyzed by HPLC-MS and the ACE-inhibitory activity and protein content were determined. As shown in Table 1, hydrolysis of caseins with pepsin under our conditions occurred rapidly, giving a protein content in the soluble fraction of 0.45% (w/v) after 1 h of hydrolysis. The ACE-inhibitory activity of the soluble fraction increased during the first 3 h of hydrolysis and the lowest value for the IC_{50} value (22.19 μg mL^{-1}), i.e., the maximum activity, was reached after 3 h hydrolysis. This IC_{50} value was comparable to the activity found in other previously reported antihypertensive protein hydrolysates obtained by enzymatic digestion or by fermentation (Miguel et al., 2004; Muguerza et al., 2006).

3.2. Identification of ACE-inhibitory peptides

The water-soluble fraction of the 3h-hydrolysate with pepsin was first subjected to ultrafiltration through a 3000 Da cut-off membrane and the ACE-inhibitory activity of the permeate and the retentate was measured. The activity of the 3000 Da permeate (IC_{50} 5.68 ± 0.36 μg mL^{-1}) was four times higher than that found in the total water-soluble extract (IC_{50} 22.19 ± 1.61 μ μg mL^{-1}) and 40 times higher than the activity measured in the retentate fraction (IC_{50} 231 ± 35 μg mL^{-1}).

The 3000 Da permeate was subjected to semi-preparative RP-HPLC. The 70-min chromatogram was divided into 8 fractions, which were collected, concentrated by freeze-drying. These fractions were dissolved in Milli-Q water at concentrations between 2.7-6 mg

213 mL^{-1}, and then, their ACE-inhibitory activity was measured (Figure 1A and 1B). Two
214 fractions, F3 and F5 in Figure 1, exhibited the highest ACE-inhibitory activities, with IC_{50}
215 values of 5.65± 0.54 μg mL^{-1} and 5.51± 0.60 μg mL^{-1}. The other three chromatographic
216 fractions, F2, F4 and F6, also showed notable potent ACE-inhibitory activity with IC_{50} values
217 under 14 μg mL^{-1}. All these active chromatographic fractions (F2, F3, F4, F5 and F6 in Fig. 1)
218 were analysed by HPLC-MS/MS. As an example, Fig. 2A shows the MS/MS spectrum of ion
219 *m/z* 905.5 that matched with the α_{s1}-CN-derived peptide f(21-31), with sequence FVAPFPEV.
220 This spectrum contained a major ion at *m/z* 588.2, which was identified as a *y*-type fragment
221 ion resulting from the cleavage N-adjacent to proline (y_5). It is known that the presence of
222 proline residues in a peptide has a significant effect upon the fragments observed by MS/MS:
223 the fragment ions produced N-terminally to proline are over-represented in the spectra
224 (Papayannopoulos, 1995). The fragment ions corresponding to the fragmentation N-terminal
225 to the other proline residue, i.e., b_5 and y_3, occurred at lower abundance than y_5, although
226 similar X-P relative bond cleavage ratios have been reported when X was alanine or
227 phenylalanine (Breci, Tabb, Yates, & Wysocki, 2003). The MS/MS spectrum in Fig. 2B was
228 consistent with the sequence AYFYPEL that corresponds to fragment (143-149) from α_{s1}-CN.
229 In this spectrum, the most prominent fragment ions were y_3 and b_4, which corresponded to
230 cleavage N-adjacent to proline.

231 The major peptide components of each active HPLC fraction were identified and these
232 results are summarised in Table 2. A total of 44 peptide sequences were identified of which
233 13 peptides corresponded to β-casein (β-CN) fragments, and 19 to α_{s1}-CN fragments. The
234 most abundant peptides in each chromatographic fraction are given in bold in Table 2. Among
235 the identified peptides, one of them had previously been described as an ACE inhibitor. More
236 specifically, the α_{s1}-CN peptide DAYPSGAW, had been found to exhibit ACE-inhibitory
237 activity with an IC_{50} value of 98 μM (Pihlanto-Leppälä, Rokka, & Korhonen, 1998). Other
238 peptides show high homology with ACE-inhibitory peptides previously described in the

239 literature, such as, NLLRF which share five residues with ENLLRF, and that has an IC_{50}
240 value of 82.4 μM (Quirós, Hernández-Ledesma, Ramos, Amigo, & Recio, 2005). Similarly,
241 peptide VQVTSTAV includes the sequence VTSTAV (IC_{50}, 52 μM) (Schlothauer et al.,
242 1999), and peptide LQY, found in fraction 2, corresponds to the C-terminal tripeptide of
243 FPQYLQY (IC_{50}, 14 μM) (Tauzin, Miclo, & Gaillard, 2002). However, the presence of these
244 peptides with moderate ACE-inhibitory activity could not explain the high activity found in
245 the permeate from the casein hydrolysate and in the isolated fractions.
246
247 *3.3. ACE-inhibitory, antioxidant and antihypertensive activity of the synthetic peptides*
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249 Several peptides, among the most abundant in each chromatographic fraction, were
250 selected on the basis of their sequences, especially the C-terminal tripeptide. A total of six
251 peptides were chemically synthesized and the ACE-inhibitory, calculated as IC_{50} value, was
252 measured (Table 3). Because it has been reported that antioxidant activity of several
253 antihypertensive peptides can also contribute to their activity (Duffy et al., 1999), the radical
254 scavenging activity of these synthetic sequences was also evaluated. Three of the six peptides,
255 with sequences RYLGY, AYFYPEL and YQKFPQY, exhibited potent ACE-inhibitory
256 activity with IC_{50} values lower than 20 μM (Table 3). Interestingly, these three peptides also
257 had high radical scavenging activity with values at least two times higher than Trolox. The
258 two α_{s1}-casein-derived peptides, i.e., RYLGY and AYFYPEL, were among the most
259 abundant in the hydrolysate and the 3000 Da-permeate fraction obtained from it, and
260 therefore, given their activity (IC_{50} values of 0.71 and 6.58 μM, respectively) and
261 concentration, they might be responsible for the activity found in the total hydrolysate.
262 The antihypertensive effect of the three peptides with higher ACE-inhibitory potency
263 was examined in SHR. Animals used for the experiment had an average basal SBP of 178±4
264 mm Hg. Fig. 3 shows the time-course changes in SBP after oral administration of the

synthetic peptides and after the administration of zofenopril (positive control). All the peptides displayed antihypertensive effects. Peptide RYLGY lowered SBP in a progressive and maintained manner during most of the time of study. The maximum reduction, observed 6 h post-administration, was 25±3 mm Hg (Fig. 3B). Peptide AYFYPEL showed a significant effect (Fig. 3A) but a smaller and less maintained effect than this was reached with the same dose of RYLGY. A small and short effect was observed with YQKFPQY (Fig. 3C). The maximum antihypertensive effect of VPP (20±5 mm Hg; Fig. 3D) was similar to that of AYFYPEL, but none of the peptides reached the decrease in SBP caused by zofenopril (Fig. 3E).

4. Discussion

In this study we have identified three novel casein-derived sequences with potent ACE-inhibitory activity, and antioxidant activity; two of them exerting high antihypertensive activity in SHR. The most potent ACE-inhibitory peptides found in this hydrolysate, with sequences RYLGY, AYFYPEL and YQKFPQY, are reported as antihypertensive peptides for the first time. Other peptides related to these had previously been found in protein hydrolysates. For instance, peptide YFYPEL had been found in a simulated gastrointestinal digestion of an infant formula (Hernández-Ledesma, Quirós, Amigo, & Recio, 2007c) and Suetsuna, Ukeda, and Ochi (2000) had reported high radical scavenging activity for this peptide. The peptide AYFYPE had previously been identified in an hydrolysate produced with an extracellular protease of *Lactobacillus helveticus* but this peptide, lacking leucine at the C-terminal end in comparison with AYFYPEL, only exhibited moderate ACE-inhibitory activity (IC_{50} 106 μM) (Yamamoto, Akino, & Takano, 1994). The α_{s2}-CN-derived peptide, YQKFPQY, had been found in a chromatographic fraction with ACE-inhibitory activity derived from Yak milk caseins hydrolysed with Neutrase® (Jiang, Chen, Ren, Luo, & Zeng,

291 2007) but the activity of the purified peptide had not been tested. Interestingly, peptide α_{s1}-
292 CN f(90-95), RYLGYL, had previously been described as a casein fragment with opioid
293 properties (Loukas, Varoucha, Zioudrou, Streaty, & Klee, 1983). If this activity is also present
294 in α_{s1}-CN f(90-94), RYLGY, it could contribute, in addition to the ACE-inhibitory activity, to
295 the antihypertensive effect exerted by this peptide. It has been found that several opioid
296 peptides produce a significant decrease in blood pressure of SHR and this activity may be
297 mediated by their interaction with opioid receptors (Nurminen et al., 2000). On the other
298 hand, the high radical scavenging activity found for these three peptides also probably
299 contributes to the antihypertensive effect in SHR. Antioxidant-rich diets have been shown to
300 reduce blood pressure in SHR (Akpaffiong & Taylor, 1998) and hypertensive humans (Duffy
301 et al., 1999). Captopril, a well-known potent ACE-inhibitory and antihypertensive drug, also
302 exhibits antioxidant properties (Gurer, Neal, Yang, Oztezcan, & Ercal, 1999). This dual
303 (ACE-inhibitory and antioxidant) activity has also been demonstrated for other milk-derived
304 peptides (Hernández-Ledesma, Amigo, Recio, & Bartolomé, 2007a).

305 Among the ACE-inhibitory peptides studied (Table 3), RYLGY and
306 YQKFPQY contained the amino acid Tyr as C-terminal residue. YQKFPQY contains Pro as
307 the antepenultimate amino acid and this residue in this position has been demonstrated to
308 enhance binding to the enzyme (Gómez-Ruiz, Ramos, & Recio, 2004; Quirós et al., 2007;
309 Rohrbach, Williams, & Rolstad, 1981). Surprisingly, AYFYPEL was the second most active
310 peptide although it contains a Glu residue in the penultimate C-terminal position. This is also
311 in accordance with the high activity of peptides derived from α-lactalbumin with C-terminal -
312 PEW as previously reported (Otte, Shalaby, Zakora, & Nielsen, 2007a). However,
313 FVAPFPEV showed low ACE-inhibitory activity verifying that the amino acid Leu probably
314 contributes significantly to the activity (Gómez-Ruiz et al., 2004). The importance of the
315 position of the amino acid proline is also demonstrated in peptide HLPLPLL where proline is
316 situated in the antepenultimate position, which favors binding to ACE (IC_{50} 34.4 μM). This

317 result agrees with the IC_{50} value reported for NLHLPLPLL, IC_{50} 15 μM, which was found in a
318 hydrolysate of sodium caseinate with *Lactobacillus helveticus* NCC 2765 (Robert, Razaname,
319 Mutter, & Juillerat, 2004).
320
321 **5. Conclusions**
322
323 The hydrolysis of isoelectric casein with pepsin generates antihypertensive peptides
324 and the peptides responsible of this activity have been identified. The most potent ACE-
325 inhibitory peptides found in this hydrolysate corresponded to the sequences RYLGY,
326 AYFYPEL, and YQKFPQY. These three sequences have in vitro radical scavenging activity
327 and also exerted significant antihypertensive activity when administered orally at SHR at
328 doses of 5 mg kg^{-1} of body weight. It must be highlighted that the antihypertensive effect
329 found for peptides RYLGY and AYFYPEL is comparable to the activity of VPP, an
330 antihypertensive peptide already included in functional foods. These sequences together with
331 their antihypertensive activity has been demonstrated here for the first time. Further studies
332 to evaluate resistance of these sequences to gastrointestinal enzymes and chronic
333 administration of the total hydrolysate in SHR is already in progress.
334
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336
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Figure legends

Fig. 1. Fractionation (A) by RP-HPLC at semi-preparative scale of the 3000 Da-permeate obtained from the peptic hydrolysate of bovine casein and (B) angiotensin-converting enzyme-inhibitory activity, expressed as IC_{50} value, of the collected fractions. Collected fractions are termed with F followed with a number (F1-F8). For the IC_{50} determination, protein content of the fractions collected from the HPLC was estimated by the bicinchoninic acid assay. Data are expressed as mean ± SEM for a minimum of three experiments.

Fig. 2. Tandem mass spectrum of (A) the singly charged ion *m/z* 905.5 and of (B) singly charged ion *m/z* 902.5, both included in fraction 5. Following sequence interpretation and data base searching, the peptides were identified as α_{s1}-casein f(24-31) and α_{s1}-casein f(143-149), respectively. The sequences of these peptides are displayed with the fragment ions observed in the spectra.

Fig. 3. Change in systolic blood pressure (SBP) after oral administration of different peptides (5 mg kg^{-1}): (A), peptide AYFYPEL; (B), peptide RYLGY; (C), peptide YQKFPQY; (D), peptide VPP; (E), peptide ZOFENOPRIL. Closed symbols are controls, open symbols are the papetides. Data are expressed as mean ± SEM for a minimum of four animals (n=4-8). Control groups received the same volume of water. Unpaired student's t-test was used to compare groups: **P*< 0.05, versus control group, ***P*< 0.01 versus control group.

Table 1.

Angiotensin-converting enzyme-inhibitory activity, expressed as the protein concentration required to inhibit the enzyme activity by 50% (IC_{50}) of the supernatants from the casein hydrolysates prepared with pepsin. Activity expressed as mean ± standard error (n= 3)

Hydrolysis time	IC_{50} (μg protein mL^{-1})	Protein (%, w/v)
1 h	60.85 ± 10.8	0.45
2 h	35.27 ± 3.6	0.45
3 h	22.19 ± 1.6	0.45
4 h	29.65 ± 3.9	0.46
5 h	38.85 ± 3.7	0.47
6 h	45.48 ± 10.1	0.46
7 h	37.07 ± 2.8	0.47
24 h	25.68 ± 1.6	0.47

Table 2.
Identification of peptides contained in the RP-HPLC fractions with angiotensin-converting enzyme-inhibitory activity.

Fraction[a]	Ion for MS/MS (*m/z*)[b]	Calculated mass	Observed mass[c]	Protein fragment	Sequence[d]
F2	675.1 (1)	674.32	674.1	β-CN f(1-5)	RELEE
	795.2 (1)	794.35	794.2	β-CN f(118-124)	PFTESQS
	763.2 (1)	762.36	762.2	α_{S1}-CN f(56-61)	DIKQME
	423.1 (1)	422.22	422.1	α_{S2}-CN f(96-98)	**LQY**
	804.3 (1)	803.44	803.3	κ-CN f(162-169)	**VQVTSTAV**
	521.2 (1)	520.27	520.2	α_{S2}-CN f(85-88)	INQF
	628.3 (1)	627.32	627.3	β-CN f(6-11)	**LNVPGE**
	502.2 (1)	501.28	501.2	α_{S1}-CN f(95-98)	**LEQL**
	423.2 (1)	422.22	422.2	α_{S1}-CN f(154-156)	YQL
F3	597.5 (1)	596.31	596.5	α_{S1}-CN f(150-153)	FRQF
	671.5 (1)	670.34	670.5	α_{S1}-CN f(90-94)	**RYLGY**
	973.5 (1)	972.47	972.5	α_{S2}-CN f(89-95)	YQKFPQY
	800.4 (1)	799.34	799.4	α_{S1}-CN f(173-179)	YTDAPSF
	866.4 (1)	865.37	865.4	α_{S1}-CN f(157-164)	**DAYPSGAW**
	940.5 (1)	939.48	939.5	α_{S1}-CN f(165-172)	**YYVPLGTQ**
	465.3 (1)	464.26	464.3	α_{S1}-CN f(92-95)	LGYL
	890.5 (1)	889.48	889.5	κ-CN f(138-146)	AVESTVATL
F4	787.3 (1)	786.43	786.3	β-CN f(157-163)	FPPQSVL
	784.3 (1)	783.43	783.3	α_{S1}-CN f(90-95)	**RYLGYL**
	1001.3 (1)	1000.51	1000.3	α_{S1}-CN f(150-156)	**FRQFYQL**
	662.3 (1)	661.39	661.3	α_{S1}-CN f(19-23)	**NLLRF**
	831.2 (1)	830.39	830.2	α_{S1}-CN f(144-149)	**YFYPEL**
	1198.4 (1)	1197.62	1197.4	κ-CN f(96-105)	ARHPHPHLSF
F5	957.4 (2)	1912.91	1912.8	β-CN f(141-156)	QSWMHQPHQPLPPTVM
	1422.8 (1)	1421.79	1421.8	κ-CN f(18-29)	FSDKIAKYIPIQ
	617.4 (1)	617.32	616.4	κ-CN f(53-57)	NQFLP
	1219.6 (1)	1218.63	1218.6	α_{S1}-CN f(25-35)	VAPFPEVFGKE
	729.5 (1)	728.40	728.5	β-CN f(199-205)	GPVRGPF
	902.5 (1)	901.42	901.5	α_{S1}-CN f(143-149)	**AYFYPEL**
	845.6 (1)	844.44	844.6	κ-CN f(51-57)	**INNQFLP**
	905.5 (1)	904.47	904.5	α_{S1}-CN f(24-31)	**FVAPFPEV**
	1063.4 (2)	2124.12	2124.0	κ-CN f(51-68)	INNQFLPYPYYAKPAAVR
F6	919.4 (2)	1835.95	1836.8	α_{S1}-CN f(24-39)	**FVAPFPEVFGKEKVNE**
	1215.6 (2)	2429.27	2429.2	β-CN f(59-80)	VYPFPGPIHNSLPQNIPPLTQT
	1151.7 (1)	1150.69	1150.7	β-CN f(199-209)	**GPVRGPFPIIV**
	1094.7 (1)	1093.66	1093.7	β-CN f(200-209)	PVRGPFPIIV
	802.6 (1)	801.51	801.6	β-CN f(134-140)	HLPLPLL
	905.5 (1)	904.47	904.5	α_{S1}-CN f(25-32)	**VAPFPEVF**
	995.6 (1)	994.59	994.6	β-CN f(81-89)	PVVVPPFLQ
	1300.6 (1)	1299.69	1299.6	β-CNA[2] f(59-70)	VYPFPGPIPNSL
	1008.6 (1)	1007.58	1007.6	κ-CN f(25-32)	YIPIQYVL
	527.2 (1)	526.35	526.2	κ-CN f(46-50)	KPVAL
	742.6 (1)	741.44	741.6	β-CN f(203-209)	**GPFPIIV**
	1015.5 (1)	1014.51	1014.5	α_{S1}-CN f(142-149)	LAYFYPEL

[a] Fractions are termed as in Fig. 1.
[b] Charge is given in parenthesis
[c] Calculated monoisotopic mass
[d] Amino acids are designed using one letter code. Most abundant peptides in each fraction are given in bold.

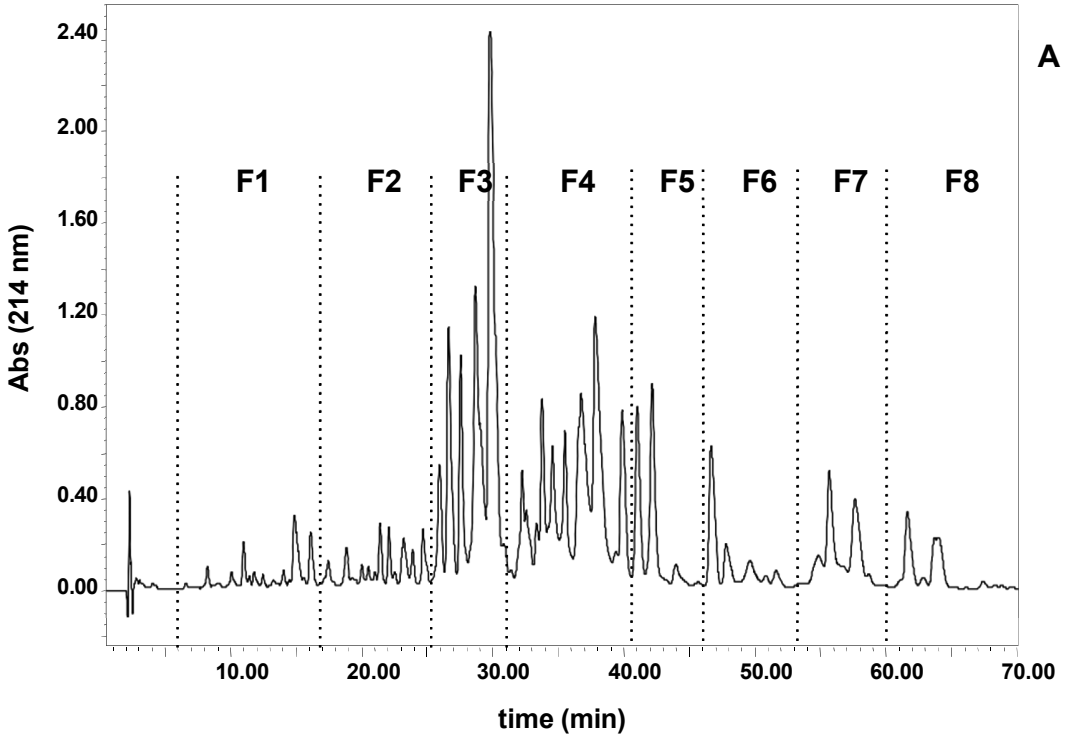
Table 3.

Angiotensin-converting enzyme-inhibitory activity and radical scavenging activity of synthetic peptides selected from those identified in the active chromatographic fractions.

Sequence	Protein fragment	IC_{50}[a] ± SE (μM)	ORAC-FL ± SEM[b] (μmol equiv Trolox μmol^{-1} peptide)
RYLGY	α_{S1}-CN f(90-94)	0.71 ± 0.08	2.829 ± 0.040
AYFYPEL	α_{S1}-CN f(143-149)	6.58 ± 0.50	3.216 ± 0.114
YQKFPQY	α_{S2}-CN f(89-95)	20.08 ± 1.25	2.033 ± 0.077
HLPLPLL	β-CN f(134-140)	34.40 ± 2.22	0.060 ± 0.002
VAPFPEVF	α_{S1}-CN f(25-32)	362.50 ± 23.56	0.046 ± 0.001
FVAPFPEV	α_{S1}-CN f(24-31)	475.89 ± 41.73	< 0.025

[a] IC_{50} value corresponds to the concentration of peptide needed to inhibit 50% of the original ACE activity, expressed as mean ± standard error (n= 3).

[b] Standard error of the mean (n=3).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Abs (214 nm)
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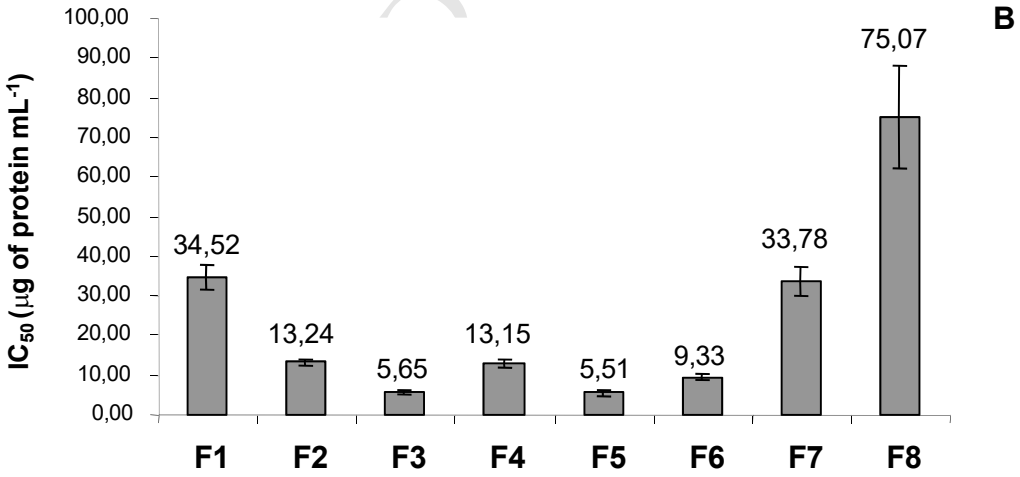
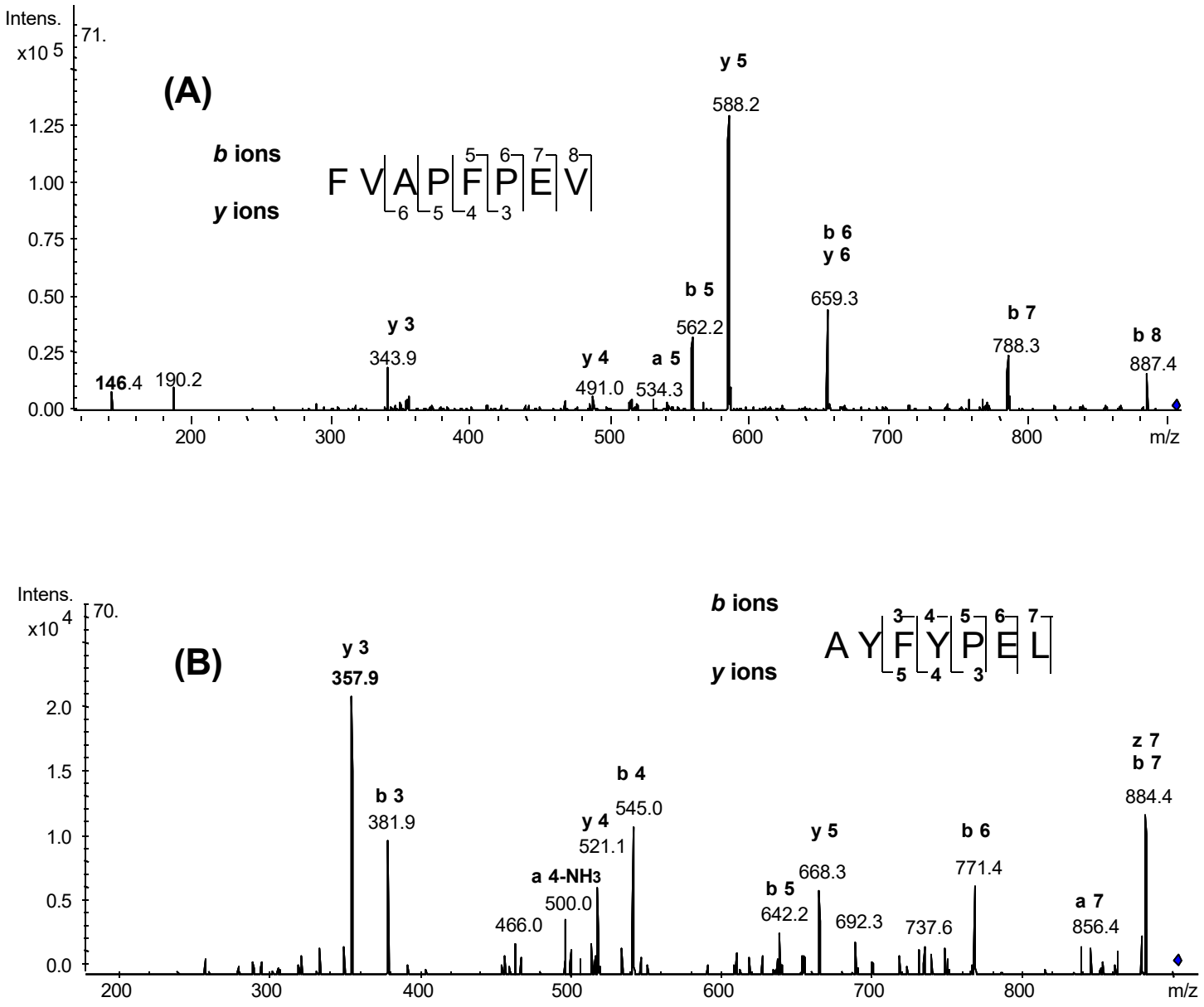
2.00
1.60
1.20
0.80
0.40
0.00
F1
F2
F3
F4
F5
F6
F7
F8
10.00
20.00
30.00
40.00
50.00
60.00
70.00
time (min)


B
IC50 (μg of protein mL-1)
100,00
90,00
80,00
70,00
60,00
50,00
40,00
30,00
20,00
10,00
0,00
34,52
13,24
5,65
13,15
5,51
9,33
33,78
75,07
F1
F2
F3
F4
F5
F6
F7
F8


Intens.
x10 5
71.
(A)
b ions
y ions
F V A P F P E V
5 6 7 8
6 5 4 3
y 5
588.2
b 6
y 6
659.3
b 5
562.2
y 3
343.9
y 4
491.0
a 5
534.3
b 7
788.3
b 8
887.4
146.4
190.2
1.25
1.00
0.75
0.50
0.25
0.00
200
300
400
500
600
700
800
m/z
Intens.
x10 4
70.
(B)
b ions
y ions
A Y F Y P E L
3 4 5 6 7
5 4 3
y 3
357.9
b 3
381.9
b 4
545.0
y 4
521.1
a 4-NH3
500.0
466.0
y 5
668.3
b 5
642.2
692.3
737.6
b 6
771.4
a 7
856.4
z 7
b 7
884.4
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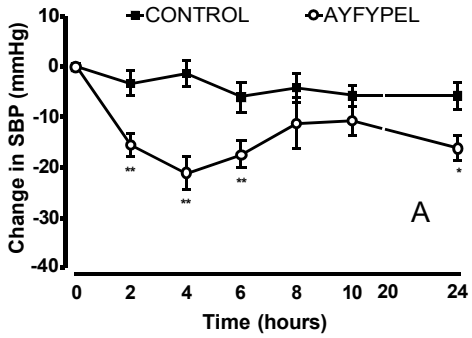
1.5
1.0
0.5
0.0
200
300
400
500
600
700
800
m/z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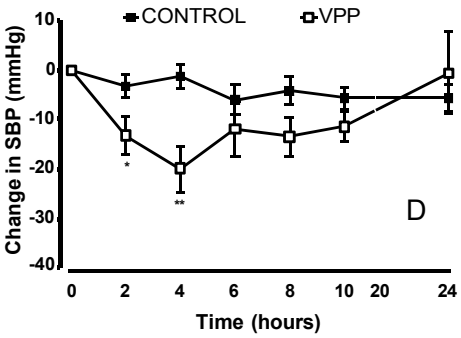
-20
-30
-40
0 2 4 6 8 10 20 24
Time (hours)
A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Change in SBP (mmHg)
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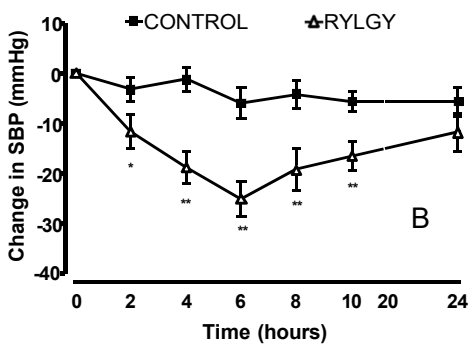
-20
-30
-40
0 2 4 6 8 10 20 24
Time (hours)
D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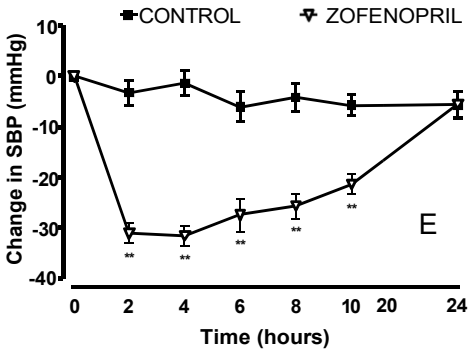
-30
-40
0 2 4 6 8 10 20 24
Time (hours)
B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ZOFENOPRIL
Change in SBP (mmHg)
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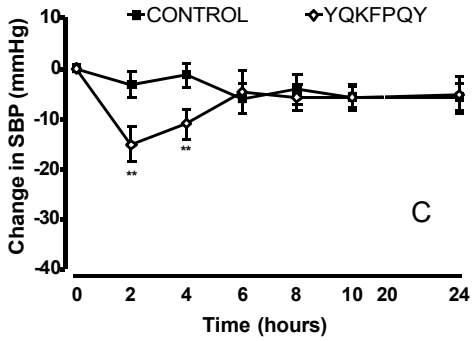
0
-10
-20
-30
-40
0 2 4 6 8 10 20 24
Time (hours)
E


CONTROL
YQKFPQY
Change in SBP (mmHg)
10
0
-10
-20
-30
-40
0 2 4 6 8 10 20 24
Time (hours)
C