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Novel Antihypertensive Lactoferrin-Derived Peptides Produced by *Kluyveromyces marxianus*: Gastrointestinal Stability Profile and *in vivo* Angiotensin I-Converting Enzyme (ACE) Inhibition

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Abstract

Novel antihypertensive peptides released by *Kluyveromyces marxianus* from bovine lactoferrin (LF) have been identified. *K. marxianus* LF permeate was fractionated by semi-preparative high performance liquid chromatography and 35 peptides contained in the angiotensin I converting enzyme (ACE)-inhibitory fractions were identified by using an ion trap mass spectrometer. Based on peptide abundance and common structural features, six peptides were chemically synthesized. Four of them (DPYKLRP, PYKLRP, YKLRP and GILRP) exerted *in vitro* inhibitory effects on ACE activity and effectively decreased systolic blood pressure after oral administration to spontaneously hypertensive rats (SHRs). Stability against gastrointestinal enzymes suggested that the sequence LRP could contribute to the *in vivo* effects of parental peptides. Finally, there were reductions in circulating ACE activity and angiotensin II level in SHRs after either DPYKLRP or LRP intake, thus confirming ACE inhibition as *in vivo* mechanism for their antihypertensive effect.

Keywords: *Kluyveromyces marxianus*, lactoferrin-derived peptides, gastrointestinal digestion, antihypertensive effect, *in vivo* ACE inhibition.

INTRODUCTION

In the last decade much work has been done to characterize the antihypertensive effects of peptides derived from food proteins.[1] Angiotensin I-converting enzyme (ACE) inhibition is the main target for those peptides. ACE, as part of the renin-angiotensin system (RAS), hydrolyzes both the inactive angiotensin I into vasoconstrictor angiotensin II and the vasodilator bradykinin into an inactive peptide leading to blood pressure upregulation.[2] *In vitro* inhibitory effect of food protein derived peptides on ACE activity is well established in contrast with the limited *in vivo* evidence available for the mechanism of action underlying their blood pressure lowering effect. Also bioavailability of ACE-inhibitory peptides has been intensively studied since it is known that bioactive peptides may undergo physiological transformations that determine their activity in the organism.[3] Most research has been focused on milk derived antihypertensive peptides, some of which have shown beneficial effects in clinical assays, as reported in different meta-analyses.[4]

The use of the proteolytic system of lactic acid bacteria (LAB) to hydrolyze milk proteins is a successful strategy to release antihypertensive peptides.[5] By contrast few studies exploit the proteolytic potential of yeasts despite their contribution to proteolysis in dairy products is well established. In this context, the lactose-fermenting yeast *Kluyveromyces marxianus* regularly found in milk and dairy products has been pointed out as a promise candidate to generate antihypertensive peptides from the whey proteins α-lactalbumin and β-lactoglobulin.[6] Its potential to produce fermented milk with casein-derived ACE-inhibitory peptides has been also described[7] although *in vivo* antihypertensive effects were not evaluated in any of these reports.

Bovine lactoferrin (LF), a well-characterized component of milk whey, is also a good source of antihypertensive peptides. We have shown that enzymatic LF hydrolyzates lower blood pressure and thus exhibit potential as orally effective antihypertensive compounds.[8,9] Moreover, after long-term intake of a pepsin LF hydrolyzate, there were reductions of circulating ACE activity, angiotensin II and aldosterone levels, as well as a compensatory increase of renin activity.[10] So far, only five LF-derived peptides with sequences RRWQWR, WQ[11], RPYL, LIWKL and LNNSRAP[8] have shown antihypertensive effects after oral administration to spontaneously hypertensive rats (SHRs), although based on *in silico* studies some other antihypertensive peptides are expected to be still identified and isolated from LF hydrolyzates.[12]

In a previous work, proteolytic yeast strains of *Debaryomyces hansenii*, *Kluyveromyces lactis* and *K. marxianus* isolated from cheeses[13] were screened for their ability to grow in media with LF as sole nitrogen source and to produce LF hydrolyzates containing ACE-inhibitory peptides. *K. marxianus* Km2 strain grown on LF produced the most potent hydrolyzate which, when orally administered to SHRs, exerted antihypertensive effect.[14]

The objective of the present study was to identify the LF-derived peptides produced by *K. marxianus* Km2 and characterize their antihypertensive effects. For this purpose a *K. marxianus* LF permeate enriched in peptides of molecular weight lower than 3 kDa (pLFH) was fractionated and the main peptides present in the ACE-inhibitory fractions identified by using an ion trap mass spectrometer. Selected peptides were evaluated for their inhibitory effects on ACE activity, their antihypertensive effects in SHRs and their stability against simulated

69 gastrointestinal digestion. Finally the *in vivo* effect of peptides on SHRs blood
70 ACE activity as well as angiotensin II and aldosterone levels are discussed.
71

72 **MATERIALS AND METHODS**

73 **Materials.** Bovine LF was provided by FrieslandCampina Domo (Zwolle, The
74 Netherlands). ACE from porcine kidney, captopril, and bicinchoninic acid protein
75 assay kit were purchased from Sigma (St. Louis, MO). Glucose was obtained
76 from Panreac (Barcelona, Spain), bacteriological peptone was purchased from
77 Cultimed (Barcelona, Spain) and yeast extract and agar were acquired from
78 Pronadisa (Madrid, Spain). ACE substrate o-aminobenzoylglycyl-p-
79 nitrophenylalanylproline was provided by Bachem Feinchemikalien (Bubendorf,
80 Switzerland). Corolase PP (porcine pancreatic extract) was from AB enzymes
81 (Darmstadt, Germany). Diazepam and ketamine were purchased from Roche
82 Farma (Madrid, Spain) and Parke-Davis (Alcobendas, Madrid, Spain),
83 respectively. ACE colorimetric kit was acquired from Bühlmann Laboratories
84 (Schönenbuch, Switzerland). AssayMax Angiotensin II ELISA kit was from
85 AssayPro (Saint Charles, MI) and Coat-A-Count Aldosterone ^{125}I RIA kit was
86 provided by Siemens Medical Solutions Diagnostics (Los Angeles, CA).
87

88 **Preparation of *K. marxianus* Lactoferrin Permeate (pLFH) and Fractionation**
89 **by Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC).**
90 *K. marxianus* LF hydrolyzate was prepared as previously described and it was
91 subjected to ultrafiltration through a VivaFlow 50 3kDa cut-off polyethersulfone
92 membrane (Vivascience, Sartorius Stedim Biotech, Aubagne, France). Resulting

permeate (pLFH), enriched in peptides of molecular weight lower than 3 kDa showed an IC_{50} value of 50.2 ± 2.7 μg/mL. [14]

Fractionation of pLFH was carried out by RP-HPLC using a Waters system (Waters Corporation, Milford, MA) equipped with a 1525 Binary HPLC pump, a 2996 Photodiode Array Detector and a 717 plus Autosampler in combination with a Fraction Collector III. For this purpose, pLFH was applied to a Prep Nova-Pak® HR C18, 60 Å, 6 μm, 7.8 x 300 mm column (Waters). The column was developed at a flow rate of 4 mL/min. Elution was performed with a linear gradient of solvent B (acetonitrile with 0.05% TFA) in solvent A (water with 0.05% TFA) from 0 to 20% B in 70 min. Samples of the whole permeate and the fractions (20 mL) were freeze-dried and kept at -20ºC until reconstitution with distilled water for determination of the protein content and *in vitro* ACE-inhibitory effect, as explained below.

Peptide Sequencing by Reversed-Phase High-Performance Liquid Chromatography Tandem Mass Spectrometry (RP-HPLC-MS/MS). RP-HPLC-MS/MS analysis of pLFH fractions was performed as described by Sánchez-Rivera et al.[15] with minor changes. The flow rate was 0.2 mL/min and the injection volume 50 μL. Peptides were eluted using a linear gradient from 0 to 45% of solvent B (acetonitrile:formic acid; 1,000:0.1, v/v) and 55% of solvent A (water:formic acid; 1,000:0.1% v/v) in 120 min. Data Analysis (version 4.0; Bruker Daltoniks) was used to process and transform spectra to representing mass values. BioTools (version 3.2; Bruker Daltoniks) was used to process the MSn spectra, to perform peptide sequencing and to calculate theoretical masses.

117 Main peptides identified in the pLFH were ordered at >90% purity from
118 GenScript Corporation (Piscataway, NJ) wherein they were synthesized by solid
119 phase methods using N-(9-fluorenyl) methoxycarbonyl (Fmoc) chemistry.
120
121 ***In vitro* Assay of ACE-Inhibitory Activity.** *In vitro* ACE-inhibitory activity of
122 pLFH fractions and synthetic peptides was measured using the fluorescent
123 method described by Sentandreu and Toldrá[16] based on the hydrolysis of the
124 internally quenched fluorescent substrate o-aminobenzoylglycyl-p-
125 nitrophenylalanylproline by the action of ACE. Protein content of peptide fractions
126 was estimated by the bicinchoninic acid method (BCA) using bovine serum
127 albumin as standard.[7] Synthetic peptide concentration was based on the dry
128 weight of the peptides.
129 The IC_{50} value was defined as the protein/peptide concentration required
130 to inhibit 50% of the ACE activity, and the value for each experiment was
131 estimated by non-linear regression of the experimental data to a four-parameter
132 logistic curve using the software package SigmaPlot v 10.0 (SPSS Inc., Chicago,
133 IL).
134
135 ***In vivo* Assay of Antihypertensive Effect in SHRs.** Experimental procedures
136 were conducted in accordance with the Spanish legislation on 'Protection of
137 Animals used for Experimental and other Scientific Purposes' and to the
138 Directives of the European Community on this subject. The study was approved
139 by the 'Ethics Committee for Animal Welfare' of 'La Fe' Hospital to be carried out
140 in its accredited animal research facility.

141 Male SHRs weighing 230–330 g (Charles River Laboratories, Barcelona,
142 Spain) were housed in temperature-controlled rooms (23°C) with 12 h light/dark
143 cycles and consumed tap water and standard diets *ad libitum*. To minimize the
144 impact of light cycle and feeding on circadian rhythms of blood pressure,[17] the
145 experiments started always at the same time in the morning (9:00 a.m.) in fasted
146 rats. Indirect measurement of systolic blood pressure (SBP) was carried out in
147 eighteen awake restrained rats by the non-invasive tail-cuff method using
148 computer-assisted Non-Invasive Blood Pressure equipment (LE5001 unit with
149 LE5160R cuff & transducer, Panlab Harvard Apparatus, Cornellá, Barcelona,
150 Spain). Peptides (up to 10 mg/kg) were orally administered by gastric intubation
151 in 650 µL of physiological saline. Before the measurements, rats were kept at
152 37°C during 15 min to make the pulsations of the tail artery detectable. The SBP
153 was measured before peptide intake (zero time) and 1, 2, 3, 4 and 24 h after
154 intake. Physiological saline (650 µL) and captopril (50 mg/kg) served as negative
155 and positive controls, respectively. Each value of SBP was obtained by averaging
156 at least three consecutive and successful measurements without disturbance of
157 the signal. Changes in SBP were calculated as the absolute difference (in mm
158 Hg) with respect to the basal values of measurements obtained just before
159 peptide administration.

160

161 ***In vitro* Simulated Gastrointestinal Digestion and Analysis of Digests by RP-**
162 **HPLC.** Peptides were subjected to a two-stage simulated gastrointestinal
163 digestion process as previously described.[10] Briefly, pepsin (0.2 mg) was added
164 to aqueous solutions of peptides (10 mL; 1 mM) adjusted at pH 2.0 using 1 N HCl
165 and incubated at 37°C. After 90 min, the pH was adjusted to 7.5 adding 10 mL of

166 0.4 M sodium phosphate buffer at pH 7.5. Corolase PP, a proteolytic enzyme
167 preparation that contains trypsin, chymotrypsin, and amino and carboxypeptidase
168 activities, was added (0.2 mg), and the sample was further incubated at 37°C for
169 150 min. The reaction was stopped by heating at 80ºC for 10 min in a water bath,
170 followed by cooling at room temperature. Each sample was stored at -20°C until
171 further analysis by RP-HPLC.

172 Analysis of gastrointestinal digests was performed in the same RP-HPLC
173 system specified above using a Symmetry C18 column (4.6 × 150 mm, 5 μm,
174 Waters) kept at 40°C. The column was developed at a flow rate of 1 mL/min.
175 Peptides were eluted with a linear gradient of solvent B (acetonitrile with 0.1%
176 TFA) in solvent A (water with 0.1% TFA) from 0 to 40% in 20 min and detected
177 at 214 nm. Peptides LRP and KLRP were quantified in gastrointestinal digests of
178 DPYKLRP, PYKLRP and YKLRP in accordance to standard curves in water.

179

180 **Determination of Blood Components of the Renin-Angiotensin System.**
181 Twenty-two rats were anaesthetized by intraperitoneal injection of 5 mg/kg
182 diazepam and 100 mg/kg ketamine. Blood samples were collected from the
183 abdominal aorta to obtain both serum and plasma which were kept frozen at -
184 80ºC until the determination of ACE activity, angiotensin II and aldosterone levels.

185 Direct quantitative *in vitro* determination of ACE activity was carried out by
186 using the Bühlmann ACE colorimetric kit according to the manufacturer's
187 instructions. Briefly, it is a kinetic enzymatic assay in which ACE catalyses the
188 cleavage of the synthetic substrate (FAPGG) into an amino acid derivative and a
189 dipeptide. The kinetic of this cleavage reaction is measured by recording the
190 decrease in absorbance at 340 nm.

191 Quantitative *in vitro* measurement of angiotensin II was carried out by
192 using the AssayMax Angiotensin II ELISA kit according to the manufacturer's
193 instructions. Briefly, this assay employs a quantitative sandwich enzyme
194 immunoassay technique in which a polyclonal antibody specific for angiotensin II
195 is pre-coated onto a microplate. The angiotensin II in standards and samples is
196 sandwiched by the immobilized antibody and biotinylated polyclonal antibody
197 specific for angiotensin II, which is recognized by a streptavidin-peroxidase
198 conjugate. A peroxidase enzyme substrate is added and intensity of developed
199 color is measured.

200 Quantitative *in vitro* measurement of aldosterone was carried out by using
201 the Coat-A-Count Aldosterone ^{125}I RIA kit according to the manufacturer's
202 instructions. Briefly, it is a solid-phase radioimmunoassay, based on aldosterone-
203 specific antibody immobilized to the wall of the assay tube. ^{125}I-labelled
204 aldosterone competes for a fixed time with aldosterone in the sample for antibody
205 sites.

206

207 **RESULTS**

208 **Fractionation of *K. marxianus* pLFH: ACE-Inhibitory Activity of Resulting**
209 **Fractions and Identification of Major Peptides.** *K. marxianus* pLFH was
210 subjected to semi-preparative RP-HPLC and the total chromatogram was divided
211 into 11 fractions which showed IC_{50} values ranging from 49 to 288 μg/mL. The
212 three most active fractions (F6, F7 and F11) with IC_{50} values of 68, 74 and 49
213 μg/mL, respectively, were analyzed by HPLC-MS/MS and the major peptide
214 components were sequenced (35 peptides on total, Table 1).

215

216 **ACE-Inhibitory Activity of LF-Derived Peptides.** A total of 6 peptides (labeled
217 in Table 1) from those identified in fractions F6, F7 and F11 were chemically
218 synthesized. These included four sequences (DGKEDL, ESPQTHY, YKLRP and
219 DPYKLRP) that being among the most abundant in each fraction also fulfilled the
220 common structural features described for many ACE-inhibitory peptides derived
221 from food proteins.[18] Since the role of specifically C-terminal P residue in
222 enhancing inhibition has been highlighted in most effective antihypertensive
223 sequences derived from milk proteins,[1] the peptides PYKLRP and GILRP
224 identified in the most active fraction (F11) were also included in the study despite
225 not being abundant. Interestingly the yeast proteolytic system produced the set
226 of sequences DPYKLRP, PYKLRP and YKLRP differing in the amino acidic
227 residue at the N-terminal end. With the aim of establishing sequence-inhibitory
228 potency relationships, the peptides KLRP and LRP were also synthesized.

229 Only the six peptides having a P residue at the C-terminal end showed
230 detectable inhibitory activity at 20 μM under our *in vitro* assay conditions. Further
231 concentration response curves allowed the determination of IC_{50} values (Table
232 2) which varied over a 200-fold range. The higher potency as indicated by lower
233 IC_{50} value corresponded to the tripeptide LRP.

234

235 **Antihypertensive Effect of LF-Derived Peptides.** The antihypertensive effect
236 of the six ACE-inhibitory peptide sequences was characterized in SHRs. Average
237 SBP, measured by the tail-cuff method in awake SHRs, was 200 ± 1 mm Hg (n =
238 58). Oral administration of the six LF-derived peptides at 10 mg/kg induced
239 significant reductions in SBP as shown in Figure 1, together with the lack of effect
240 of oral saline and the antihypertensive effect of captopril (50 mg/kg). Similar to

241 the effect caused by captopril, the antihypertensive effect of sequences
242 DPYKLRP, GILRP and LRP remained significant up to 24 h post administration.
243 Antihypertensive effects ranged from -26.8 mm Hg for both DPYKLRP and LRP
244 till -13.2 mm Hg for KLRP. Reductions in SBP caused by DPYKLRP (-26.8 ± 2.4
245 mm Hg; 1 h post administration) and LRP (-26.8 ± 1.3 mm Hg; 2 h) were
246 comparable to that of the captopril control (-27.9 ± 2.1 mm Hg; 1 h) (one-way
247 ANOVA; $P>0.05$).

248 The heptapeptide DPYKLRP and the tripeptide LRP were further studied
249 for dose-dependent antihypertensive effects. Both peptides induced significant
250 dose-dependent (3, 7 and 10 mg/kg) reductions in SBP at each time point from 1
251 h to 24 h after oral administration (Figure 2).

252

253 **Resistance of LF-Derived Peptides to Gastrointestinal Enzymes.** The six
254 antihypertensive peptides were subjected to a hydrolysis process which
255 simulates gastrointestinal digestion due to the action of gastric and pancreatic
256 enzymes. The analysis of digests by RP-HPLC (Figure 3) showed that the longer
257 sequences, DPYKLRP and PYKLRP, were completely hydrolyzed releasing
258 several fragments. A partial hydrolysis was observed for the pentapeptide YKLRP
259 (approximately 60% of the initial concentration of the input peptide). In the
260 conditions tested, sequences KLRP and GILRP were slightly hydrolyzed
261 (approximately 6% and 12% decrease from the initial concentrations) whereas
262 LRP was resistant to gastrointestinal enzymes. Noteworthy, in the
263 gastrointestinal digests of the hydrolyzed peptides, the sequences LRP and
264 KLRP were detected among others. LRP at concentrations of 525 μM, 600 μM
265 and 465 μM were detected in the digests of DPYKLRP, PYKLRP and YKLRP,

266 respectively. Also a minor quantity of LRP (3 μM) was detected in the KLRP
267 digest. In the conditions tested, the sequence LRP was not detected in the GILRP
268 digest. With respect to KLRP, concentrations of 550 μM and 140 μM were
269 detected in the digests of DPYKLRP and PYKLRP. Also the sequence KLRP was
270 detected at a concentration of 17 μM in the YKLRP digest.

271

272 **Effects of LF-Derived Peptides on Blood Components of the Renin-**
273 **Angiotensin System.** The effects of DPYKLRP and LRP (10 mg/kg) on serum
274 ACE activity and angiotensin II levels, and on plasma aldosterone levels were
275 studied in SHRs. Captopril (50 mg/kg) was also included as a positive control.

276 The average serum ACE activity for all measurements carried out in the
277 three experimental groups before treatment intake was 111.4 ± 1.8 U/L (n=22).
278 As shown in Figure 4A, ACE activity was significantly reduced in SHRs treated
279 with DPYKLRP, LRP and captopril at 1 h and 4 h post administration, and
280 reverted to initial values after 24 h. At 1 h post administration, when maximum
281 effects were observed, the reduction in ACE activity induced by DPYKLRP (48.1
282 ± 2.5%) was similar to that caused by captopril (43.4 ± 3.1%), and significantly
283 higher than the reduction induced by LRP (19.1 ± 2.7%) in SHRs (one way
284 ANOVA followed by Student-Newman-Keuls test).

285 SHRs showed an average serum angiotensin II level of 71.2 ± 1.3 pg/mL
286 (n=22) before treatment intake. Angiotensin II levels in SHRs were significantly
287 reduced by the three treatments at 1 h post administration (Figure 4B). The effect
288 of LRP reverted at 4 h post administration whereas the reductions caused by the
289 heptapeptide and captopril reverted at 24 h. When maximum effects were
290 observed (1 h), the effects caused by DPYKLRP (27.1 ± 0.6% reduction in

291 angiotensin II levels) and captopril (33.2 ± 1.3%) were similar and higher than
292 that provoked by LRP treatment to SHRs (14.8 ± 1.9%; one way ANOVA followed
293 by Student-Newman-Keuls test).

294 By contrast to that observed in serum ACE activity and angiotensin II
295 levels, plasma aldosterone level of SHRs (244.7 ± 1.9 pg/mL; n=22) was not
296 significantly affected by any of the treatments (data not shown).

297

298 **DISCUSSION**

299 Yeast products have been used for many years as ingredients and
300 additives in food processing, although their potential bioactivity has been less
301 investigated.[19] *K. marxianus*, considered a GRAS (Generally Recognized As
302 Safe) microorganism, has been isolated from a great variety of habitats, which
303 results in a high metabolic diversity. Therefore, different biotechnological
304 applications of this yeast including production of enzymes, of single cell-protein,
305 and of aroma compounds as well as production of bioingredients from cheese-
306 whey have been described.[20] Moreover the beneficial properties of *K. marxianus*
307 as a human probiotic have been recently assessed.[21]

308 In this study, we have identified four novel LF-derived peptides which are
309 reported as ACE-inhibitory and antihypertensive sequences for the first time. To
310 the best of our knowledge, DPYKLRP, PYKLRP, YKLRP and GILRP produced
311 by the proteolytic system of *K. marxianus* Km2 strain when grown in LF as sole
312 nitrogen source, are the first peptides with antihypertensive effects after oral
313 administration to SHRs produced by a food-isolated yeast strain. Novel
314 sequences identified here could at least in part contribute to the ACE inhibiting
315 and antihypertensive effects of *K. marxianus* pLFH.[14]

316 The four *K. marxianus* ACE-inhibitory peptides have a C-terminal P
317 residue. It has been described that the rigid structure of this amino acid may lock
318 the carboxyl group into a conformation favorable for interaction with the positively
319 charged residue at the active site of the enzyme.[22] Also the four sequences share
320 the C-terminal tripeptide LRP. Interestingly LRP, which can be found in three
321 different regions of LF sequence, was pointed out as the sequence responsible
322 of the *in silico* high ACE-inhibitory activity of different peptide sequences in LF,
323 and in accordance with our results, an IC_{50} value of 0.27 μM was described for
324 the tripeptide.[12] The sequence LIWKL was the most potent LF-derived peptide
325 described so far (IC_{50} = 0.47 ± 0.01 μM).[8] Here, LRP was the most potent
326 sequence with an IC_{50} value (IC_{50} = 0.35 ± 0.03 μM) slightly lower than that of
327 LIWKL. Our results suggest that N-terminal elongations decrease *in vitro*
328 inhibitory potency, although it might not result in lower antihypertensive effects
329 (see below). Moreover elongations at the C-terminal end of the tripeptide also
330 provoked a decrease of inhibitory potency since an IC_{50} value of 4.14 μM was
331 described for the sequence LRPVAA.[23]

332 Our results in SHRs show a complex relationship between the *in vitro* ACE-
333 inhibitory potency and the *in vivo* antihypertensive effects after oral administration
334 suggesting a role for gastrointestinal digestion in the formation and degradation
335 of antihypertensive peptides. When subjected to hydrolysis with gastrointestinal
336 enzymes all of the peptides tested in this study were hydrolyzed to different
337 degrees with the exception of LRP. Remarkably this sequence was found in most
338 of the digests suggesting that the tripeptide might contribute to the *in vivo* effects
339 of parental peptides. Further work will be needed to clarify the physiological

340 relevance of LRP as well as of the other digestion fragments that could also
341 contribute to the blood pressure-lowering effects of parental peptides.

342 Although the IC_{50} values of LF-derived peptides were by far higher than
343 that of ACE-inhibitory drug captopril (0.022 μM),[24] in the conditions tested, oral
344 administration of DPYKLRP and LRP resulted in a significant decrease in SBP
345 (13.4% reduction from baseline) similar to that of captopril (14% reduction).
346 These results are also in agreement with the previously reported antihypertensive
347 effect of the LF-derived peptide LIWKL (12.1% reduction).[8] It has been reported
348 that food-derived ACE-inhibitory peptides might possess higher *in vivo* effects
349 than expected from *in vitro* inhibitory potencies due to their higher affinity to target
350 tissues and their slower elimination.[25]

351 It has been also postulated that other mechanisms of action apart from
352 ACE inhibition might underlie *in vivo* antihypertensive effect of ACE-inhibitory
353 peptides, including short-term vasoactive mechanisms as well as long term-
354 antioxidant and anti-inflammatory mechanisms.[26] In this context the sequence
355 GILRP isolated here is part of the sequences GILRPY and GILRPYL identified in
356 a proteinase K LF hydrolyzate which exerted *in vivo* antihypertensive effect. Both
357 the hydrolyzate and GILRPY showed significant endothelin converting enzyme
358 (ECE)-inhibitory effects.[9] ECE is a key peptidase in the endothelin system that
359 cleaves precursor inactive big endothelin-1 to produce active endothelin-1 which
360 has powerful vasoconstrictor and pressor properties.[27] The endothelin system
361 has an increasingly recognized role in blood pressure regulation, and has also
362 been targeted for hypertension drug treatment. Moreover, we described a set of
363 peptides derived from LF which showed inhibitory effects on ACE and ECE
364 activities.[28] Also the ACE-inhibitory peptide lactokinin can modulate endothelin-1

365 release by endothelial cells.[29] Whether the antihypertensive effect showed by
366 GILRP in this study might be also due to ECE inhibition deserves further studies.
367 Dose-dependent antihypertensive effects of DPYKLRP and LRP prompted
368 us to look for a mechanism of action responsible for the graded *in vivo* responses
369 of the LF-derived peptides. Determinations of blood RAS components support
370 ACE inhibition as an *in vivo* antihypertensive mechanism in SHRs. *In vivo* ACE-
371 inhibitory effect can be assessed by measuring tissue membrane-anchored or
372 soluble, circulating ACE activities, and confirmed by measuring circulating levels
373 of angiotensin II.[30] Our results show that serum ACE activity is reduced in SHRs
374 after oral administration of both peptides. Moreover, inhibition of ACE was
375 confirmed in peptide treated SHRs by the reduction in angiotensin II level. We
376 have previously reported that long term administration to SHRs of an
377 antihypertensive bovine LF pepsin hydrolyzate enriched in low molecular weight
378 peptides reduced circulating ACE activity, angiotensin II and aldosterone levels.[10]
379 By contrast, in the present study, the level of serum aldosterone, the adrenal
380 endocrine component downstream angiotensin II in the renin-angiotensin axis,[2]
381 was not affected by single-dose treatments with DPYKLRP and LRP. *In vivo* ACE
382 inhibition has been also pointed out as the mechanism underlying the blood
383 pressure reduction of the tripeptide IQP derived from the blue algae *Spirulina*
384 *platensis* since serum ACE and angiotensin II levels were significantly reduced in
385 SHRs after one-week treatment.[31] Nonetheless, the identification of other *in vivo*
386 mechanisms beyond ACE inhibition underlying antihypertensive effects of the LF-
387 derived peptides identified in this study should be further investigated.
388 Our results point out *K. marxianus* as a feasible GRAS microorganism for
389 the production of novel LF-derived peptides with ACE-inhibitory and

390 antihypertensive effects. The LF-derived peptides produced by *K. marxianus*,
391 DPYKLRP, PYKLRP, YKLRP and GILRP, effectively decreased arterial blood
392 pressure in SHRs and could, at least in part be responsible for the
393 antihypertensive properties previously described for *K. marxianus* LF
394 hydrolyzate. Also data reported here suggest ACE inhibition as *in vivo*
395 mechanism for the antihypertensive effects of the sequences DPYKLRP and LRP
396 in particular, although other mechanisms cannot be discarded.

397

398 **ABBREVIATIONS USED**

399 ACE, angiotensin I-converting enzyme; BCA, bicinchoninic acid method; ECE,
400 endothelin converting enzyme; LF, bovine lactoferrin; GRAS, generally
401 recognized as safe; LAB, lactic acid bacteria; pLFH, lactoferrin permeate
402 enriched in peptides of molecular weight lower than 3kDa; RAS, renin-
403 angiotensin system; RP-HPLC, reversed-phase high-performance liquid
404 chromatography; RP-HPLC-MS/MS, reversed-phase high-performance liquid
405 chromatography tandem mass spectrometry; SBP, systolic blood pressure;
406 SHRs, spontaneously hypertensive rats; TFA, trifluoroacetic acid.

407

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411

412 **References**

413 (1) Martínez-Maqueda, D.; Miralles, B.; Recio, I.; Hernández-Ledesma, B.
414 Antihypertensive peptides from food proteins: a review. *Food & Function* **2012**,
415 *3*, 350-361.
416 (2) Carey, R. M.; Siragy, H. M. (2003). Newly recognized components of the
417 renin-angiotensin system: potential roles in cardiovascular and renal regulation.
418 *Endocrine Rev.* **2003**, *24*, 261-271.
419 (3) Vermeirssen, V.; van Camp, J.; Verstraete, W. Bioavailability of angiotensin
420 I converting enzyme inhibitory peptides. *Br. J. Nutr.* **2004***, 92,* 357-366.
421 (4) Turpeinen, A. M.; Järvenpää, S.; Kautiainen, H.; Korpela, R.; Vapaatalo, H.
422 Antihypertensive effects of bioactive tripeptides-random effects meta-analysis.
423 *Ann. Med.* **2013***, 45,* 51-56.
424 (5) Hernández-Ledesma, B.; Contreras, M.M.; Recio, I. Antihypertensive
425 peptides: production, bioavailability and incorporation into foods. *Adv. Coll. Int.*
426 *Sci.* **2011**, *165*, 23-35.
427 (6) Hamme, V.; Sannier, F.; Piot, J.-M.; Didelot, S.; Bordenave-Juchereau, S.
428 Crude goat whey fermentation by *Kluyveromyces marxianus* and *Lactobacillus*
429 *rhamnosus*: contribution to proteolysis and ACE inhibitory activity. *J. Dairy Res.*
430 **2009***, 76,* 152-157.
431 (7) Chaves-López, C.; Tofalo, R.; Serio, A.; Paparella, A.; Sacchetti, G.; Suzzi,
432 G. Yeasts from colombian Kumis as a source of peptides with angiotensin-I
433 converting enzyme (ACE) inhibitory activity in milk. *Int. J. Food Microbiol.* **2012***,*
434 *159,* 39-46.
435 (8) Ruiz-Giménez, P.; Salom, J. B.; Marcos, J. F.; Vallés, S., Martínez-Maqueda,
436 D.; Recio, I.; Torregrosa, G.; Alborch, E.; Manzanares, P. Antihypertensive effect

437 of a bovine lactoferrin pepsin hydrolysate: identification of novel active peptides.
438 *Food Chem.* **2012***, 131,* 266-273.
439 (9) Fernández-Musoles, R.; Salom, J. B.; Martínez-Maqueda, D.; López-Díez,
440 J. J.; Recio, I.; Manzanares, P. Antihypertensive effects of lactoferrin
441 hydrolysates: inhibition of angiotensin- and endothelin-converting enzymes. *Food*
442 *Chem.* **2013** *139*, 994-1000.
443 (10) Fernández-Musoles, R.; Manzanares, P.; Burguete, M. C.; Alborch, E;
444 Salom J. B. *In vivo* angiotensin I-converting enzyme inhibition by long-term intake
445 of antihypertensive lactoferrin hydrolysate in spontaneously hypertensive rats.
446 *Food Res. Int.* **2013**, *54*, 627-632.
447 (11) Ruiz-Giménez, P.; Ibáñez, A.; Salom, J. B.; Marcos, J. F.; López-Díez, J.
448 J.; Vallés, S.; Torregrosa, G.; Alborch, E.; Manzanares, P. Antihypertensive
449 properties of lactoferricin B-derived peptides. *J. Agric. Food Chem.* **2010***, 58,*
450 6721-6727.
451 (12) Vermeirssen, V.; van der Bent, A.; van Camp, J.; van Amerongen, A.;
452 Verstraete, W. A quantitative *in silico* analysis calculates the angiotensin I
453 converting enzyme (ACE) inhibitory activity in pea and whey protein digests.
454 *Biochim.* **2004***, 86*, 231-239.
455 (13) Padilla, B.; Manzanares, P.; Belloch, C. (2014). Yeast species and genetic
456 heterogeneity within *Debaryomyces hansenii* along the ripening process of
457 traditional ewes' and goats' cheeses. *Food Microbiol.* **2014***,* 38, 160-166.
458 (14) García-Tejedor, A.; Padilla, B.; Salom, J. B.; Belloch, C.; Manzanares, P.
459 Dairy yeasts produce milk protein-derived antihypertensive hydrolysates. *Food*
460 *Res. Int.* **2013***, 53,* 203-208.

461 (15) Sánchez-Rivera, L.; Recio, I.; Ramos; M.; Gómez-Ruiz, J. A. Peptide
462 profiling in cheeses packed using different technologies. *J. Dairy Sci.* **2013***, 96,*
463 3551-3557.
464 (16) Sentandreu, M. A.; Toldrá, F. A rapid, simple and sensitive fluorescence
465 method for the assay of angiotensin-I converting enzyme. *Food Chem.* **2006***, 97*,
466 546-554.
467 (17) van den Buuse, M. Circadian rhythms of blood pressure and heart rate in
468 conscious rats: effects of light cycle shift and timed feeding. *Physiol. Behav.* **1999**,
469 *68*, 9-15.
470 (18) Murray, B. A.; FitzGerald, R. J. Angiotensin converting enzyme inhibitory
471 peptides derived from food proteins: biochemistry, bioactivity and production.
472 *Curr. Pharm. Des.* **2007**, *13*, 773-791.
473 (19) Abbas, C. A. (2006). Production of antioxidants, aromas, colours, flavours
474 and vitamins by yeasts. In *Yeasts in food and beverages*; Querol, A., Fleet, G.
475 H., Eds.; Springer: New York, NY, 2006; pp. 285-334.
476 (20) Fonseca, G. G.; Heinzle, E.; Wittmann, C.; Gombert, A. K. The yeast
477 *Kluyveromyces marxianus* and its biotechnological potential. *Appl. Microbiol.*
478 *Biotechnol.* **2008***, 79*, 339-354.
479 (21) Maccaferri, S.; Klinder, A.; Brigidi, P.; Cavina, P.; Costabile, A. Potential
480 probiotic *Kluyveromyces marxianus* B0399 modulates the immune response in
481 Caco-2 cells and peripheral blood mononuclear cells and impacts the human gut
482 microbiota in an *in vitro* colonic model system. *App. Environ. Microbiol.* **2012***, 78,*
483 956-964.

484 (22) Cushman, D. W.; Cheung, H. S.; Sabo, E. F.; Ondetti, M. A. Design of
485 potent competitive inhibitors of angiotensin-converting enzyme. Carboxyalkanoyl
486 and mercaptoalkanoyl amino acids. *Biochem.* **1977***, 16*, 5484-5491.

487 (23) Lee, N-Y.; Cheng, J-T.; Enomoto, T.; Nakamura, I. The antihypertensive
488 activity of angiotensin-converting enzyme inhibitory peptide containing in bovine
489 lactoferrin. *Chin. J. Physiol.* **2006***, 49*, 67-73.

490 (24) Ondetti, M. A.; Rubin, B.; Cushman, D. W. Design of specific inhibitors of
491 angiotensin-converting enzyme: new class of orally active antihypertensive
492 agents. *Science* **1977**, *196*, 441-444.

493 (25) Fujita, H.; Yoshikawa, M. LKPNM: a pro-drug type ACE-inhibitory peptide
494 derived from fish protein. *Immunopharmacol.* **1999***, 44*, 123-127.

495 (26) Marques, C.; Amorim, M.M.; Pereira, J.O.; Pintado, M.E.; Moura, D.;
496 Calhau, C.; Pinheiro, H. Bioactive peptides: are there more antihypertensive
497 mechanisms beyond ACE inhibition? *Curr. Pharm. Des.* **2012**, *18*, 4706-4713.

498 (27) Schiffrin, E. L. Vascular endothelin in hypertension. *Vasc. Pharmacol.* **2005**,
499 *43*, 19-29.

500 (28) Fernández-Musoles, R.; López-Díez, J. J.; Torregrosa, G.; Vallés, S.;
501 Alborch, E.; Manzanares, P.; Salom J. B. Lactoferricin B-derived peptides with
502 inhibitory effects on ECE-dependent vasoconstriction. *Peptides* **2010***, 31*, 1926-
503 1933.

504 (29) Maes W.; van Camp J.; Vermeirssen V.; Hemeryck M.; Ketelslegers J. M.;
505 Schrezenmeir J.; van Oostveldt, P.; Huyghebaert, A. Influence of the lactokinin
506 Ala-Leu-Pro-Met-His-Ile-Arg (ALPMHIR) on the release of endothelin-1 by
507 endothelial cells. *Reg. Peptides* **2004***, 118*, 105-109.

508 (30) Coates, D. The angiotensin converting enzyme (ACE). *Int. J. Biochem. Cell*
509 *Biol.* **2003**, *35*, 769-773.
510 (31) Lu, J.; Sawano, Y.; Miyakawa, T.; Xue, Y-L.; Cai, M-Y.; Egashira, Y.; Ren,
511 D-F.; Tanokura, M. One-week antihypertensive effect of Ile-Gln-Pro in
512 spontaneously hypertensive rats. *J. Agric. Food Chem.* **2011***, 59,* 559-563.
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Figure captions

Figure 1. Time course of systolic blood pressure (SBP) changes after oral administration of physiological saline, captopril (50 mg/kg) and LF-derived peptides (10 mg/kg) to SHRs. Pressure changes (ΔSBP) are expressed in absolute values (mm Hg) and data are expressed as mean ± SEM from 6-7 determinations. **P*<0.01 versus control saline group (one-way ANOVA followed by Dunnett multiple comparison tests).

Figure 2. Time course of systolic blood pressure (SBP) changes after oral administration of increasing doses of DPYKLRP (A) and LRP (B) to SHRs. (○) physiological saline, (▲) 3 mg/kg, (■) 7 mg/kg, (●) 10 mg/kg. Pressure changes are expressed in absolute values (mm Hg) and data are expressed as mean ± SEM from 5-8 determinations. Different letters indicate significant differences among doses at each time point (one-way ANOVA followed by Student-Newman-Keuls multiple comparison tests, *P*<0.05).

Figure 3. RP-HPLC chromatograms of peptides before (dashed line) and after (solid line) being submitted to a simulated gastrointestinal digestion. (A) DPYKLRP, (B) PYKLRP, (C) YKLRP, (D) KLRP, (E) LRP, (F) GILRP.

Figure 4. Time course of changes produced in the levels of blood components of the renin-angiotensin system after oral administration of DPYKLRP (10 mg/kg), LRP (10 mg/kg) and captopril (50 mg/kg) to SHRs. (A) Serum angiotensin I-converting enzyme (ACE) activity. (B) Serum angiotensin II levels. Data are mean ± SEM from 4-10 determinations. **P*<0.05 versus baseline values at time 0 h, ***P*<0.01 versus baseline values at time 0 h (one-way ANOVA followed by Dunnett multiple comparison tests).

Table 1. Identification of Peptides Contained in the F6, F7 and F11 RP-HPLC Fractions of the *K. marxianus* Lactoferrin Permeate (pLFH)

fraction[a]	ion for MS/MS (m/z)[b]	observed mass[c]	theoretical mass	protein fragment	identified sequence
F6	755.397	754.390	754.372	f(601 – 607)	SDRAAHV
	779.362	778.354	778.361	f(652 – 658)	GGRPTYE
	646.390	645.382	645.333	f(335 – 340)	AEEVKA
	676.329	675.322	675.308	f(261 – 266)	**DGKEDL**[d]
	624.367	623.360	623.303	f(283 – 287)	SRSFQ
	638.438	637.431	637.355	f(611 – 615)	LLHQQ
	668.412	667.405	667.340	f(602 – 607)	DRAAHV
	607.349	606.341	606.276	f(68 – 72)	GRDPY
	439.113	438.105	438.211	f(319 – 322)	YLGS
	722.407	721.400	721.376	f(276 – 281)	EKFGKN
F7	908.426	907.419	907.404	f(652 – 659)	GGRPTYEE
	575.214	574.206	574.260	f(536 – 541)	DVGDVA
	714.516	713.509	713.480	f(435 – 441)	AVAVVKK
	775.417	774.409	774.376	f(260 – 266)	VDGKEDL
	504.075	503.068	503.223	f(536 – 540)	DVGDV
	851.407	850.400	850.382	f(653 – 659)	GRPTYEE
	548.256	547.249	548.226	f(503 – 508)	ALCAGD
	636.454	635.447	635.375	f(338 – 342)	VKARY
	582.226	581.219	581.270	f(660 – 664)	YLGTE
	572.331	571.324	571.333	f(28 – 33)	KLGAPS
	496.155	495.148	495.244	f(283 – 286)	SRSF
	779.466	778.459	778.434	f(98 – 104)	VKKGSNF
	861.351	860.344	860.366	f(86 – 92)	**ESPQTHY**[d]
F11	848.427	847.420	847.455	f(68 – 74)	GRDPYKL
	823.472	822.465	822.387	f(224 – 229)	RDQYEL
	693.239	692.232	692.281	f(189 – 194)	YFGYSG
	743.382	742.375	742.386	f(141 – 147)	SLEPLQG
	773.499	772.492	772.460	f(71 – 76)	**PYKLRP**[d]
	907.415	906.408	906.420	f(563 – 569)	NLNREDF
	780.312	779.305	779.345	f(101 – 107)	GSNFQLD
	677.293	676.286	676.318	f(445 – 450)	GLTWNS
	676.510	675.503	675.407	f(72 – 76)	**YKLRP**[d]
	555.428	554.421	554.354	f(130 – 134)	**GILRP**[d]
	888.482	887.475	887.487	f(70 – 76)	**DPYKLRP**[d]
	414.156	413.149	413.227	f(144 – 147)	PLQG

[a]Fractions are termed as in Figure 1.
[b]Charge of precursor ion: 1
[c]Calculated monoisotopic mass
[d]Chemically synthesized peptides are labelled in bold.

546

Table 2. Inhibitory Potency of Selected LF-Derived Peptides on ACE Activity

peptide	IC_{50} (μM)[a]
DPYKLRP	30.5 ± 1.4 (c)
PYKLRP	10.2 ± 1.2 (b)
YKLRP	16.5 ± 0.7 (b)
KLRP	91.6 ± 4.0 (d)
LRP	0.35 ± 0.03 (a)
GILRP	90.7 ± 5.0 (d)

[a]Inhibitory potency is expressed as IC_{50} and data are mean ± SEM of at least 3 independent experiments. Data with the same letter are not significantly different, $P > 0.05$ (one way ANOVA followed by Student-Newman-Keuls test).

547

Figure 1

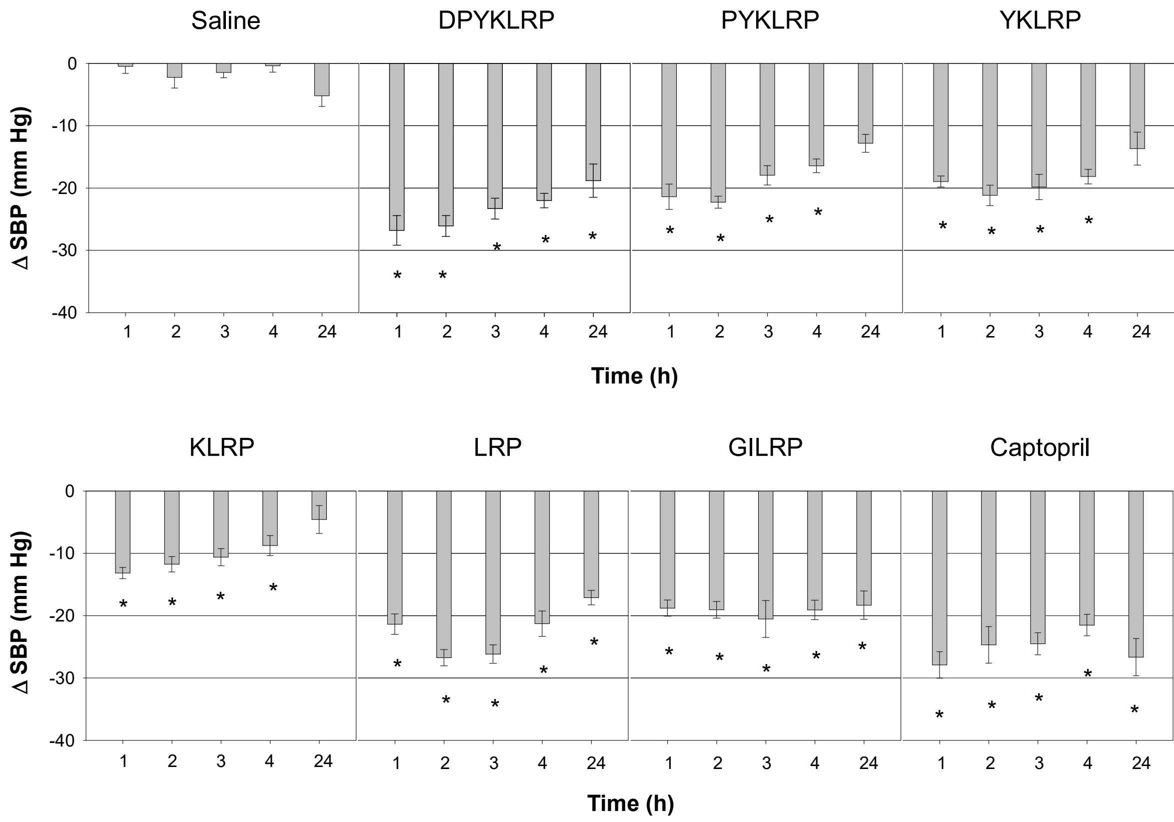

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Figure 2

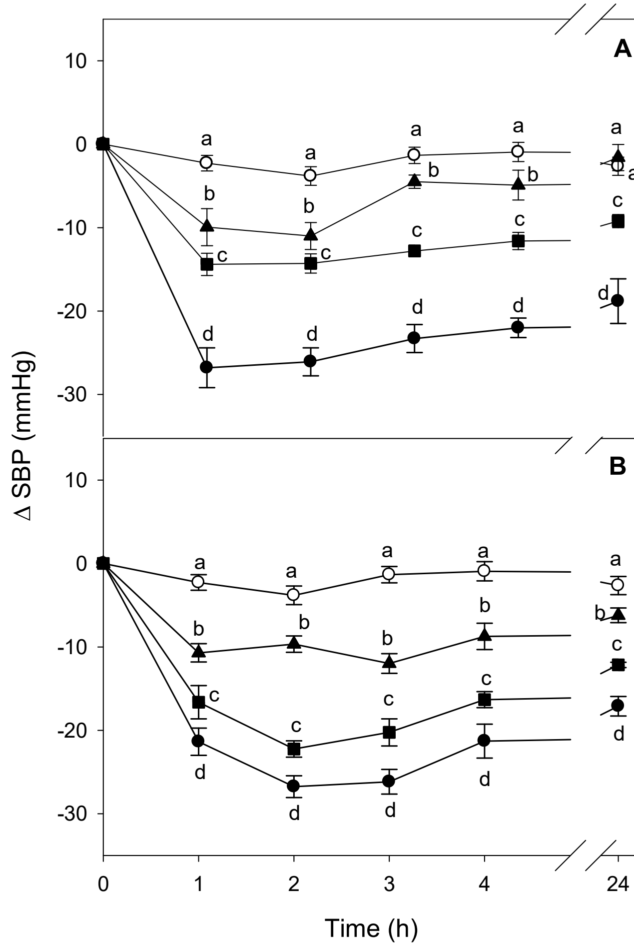

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Figure 3

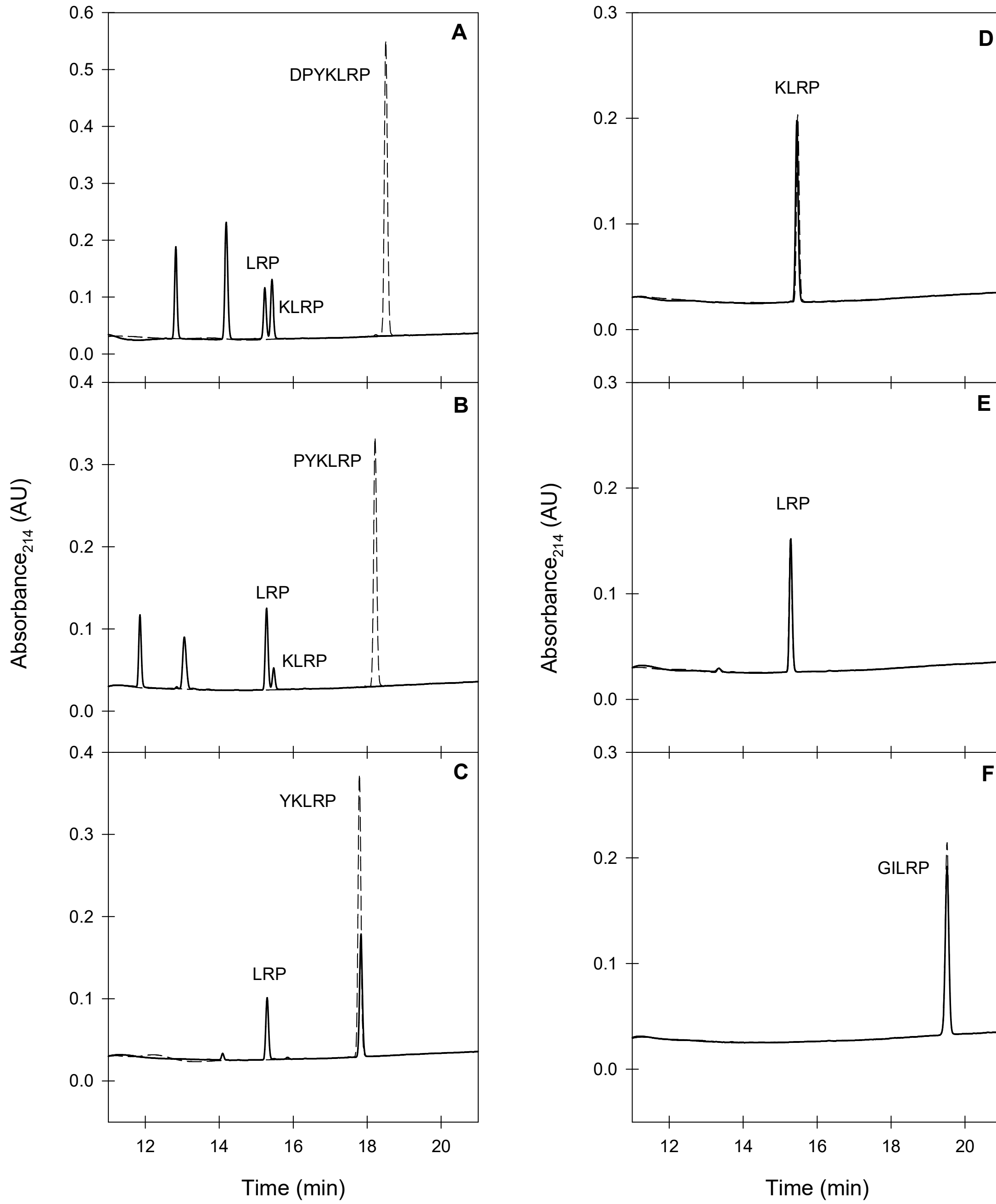

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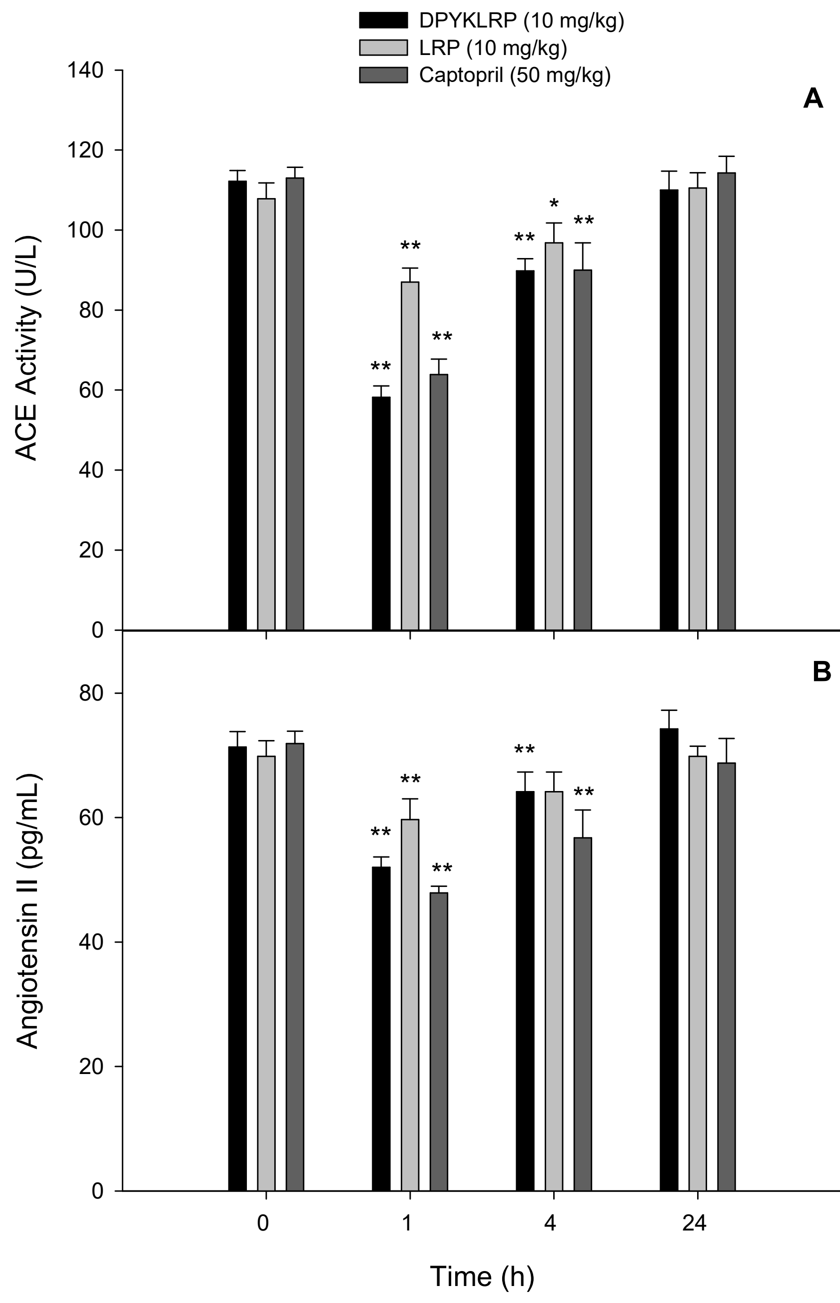
Figure 4



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