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

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

MATERIALS AND METHODS

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

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

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

95 Fractionation of pLFH was carried out by RP-HPLC using a Waters system
96 (Waters Corporation, Milford, MA) equipped with a 1525 Binary HPLC pump, a
97 2996 Photodiode Array Detector and a 717 plus Autosampler in combination with
98 a Fraction Collector III. For this purpose, pLFH was applied to a Prep Nova-Pak®
99 HR C18, 60 Å, 6 µm, 7.8 x 300 mm column (Waters). The column was developed
100 at a flow rate of 4 mL/min. Elution was performed with a linear gradient of solvent
101 B (acetonitrile with 0.05% TFA) in solvent A (water with 0.05% TFA) from 0 to
102 20% B in 70 min. Samples of the whole permeate and the fractions (20 mL) were
103 freeze-dried and kept at -20ºC until reconstitution with distilled water for
104 determination of the protein content and *in vitro* ACE-inhibitory effect, as
105 explained below.
106
107 **Peptide Sequencing by Reversed-Phase High-Performance Liquid**
108 **Chromatography Tandem Mass Spectrometry (RP-HPLC-MS/MS).** RP-
109 HPLC-MS/MS analysis of pLFH fractions was performed as described by
110 Sánchez-Rivera et al.[15] with minor changes. The flow rate was 0.2 mL/min and
111 the injection volume 50 µL. Peptides were eluted using a linear gradient from 0
112 to 45% of solvent B (acetonitrile:formic acid; 1,000:0.1, v/v) and 55% of solvent
113 A (water:formic acid; 1,000:0.1% v/v) in 120 min. Data Analysis (version 4.0;
114 Bruker Daltoniks) was used to process and transform spectra to representing
115 mass values. BioTools (version 3.2; Bruker Daltoniks) was used to process the
116 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.

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

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

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

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

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

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

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

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

RESULTS

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

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

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

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

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

release by endothelial cells.[29] Whether the antihypertensive effect showed by GILRP in this study might be also due to ECE inhibition deserves further studies.

Dose-dependent antihypertensive effects of DPYKLRP and LRP prompted us to look for a mechanism of action responsible for the graded *in vivo* responses of the LF-derived peptides. Determinations of blood RAS components support ACE inhibition as an *in vivo* antihypertensive mechanism in SHRs. *In vivo* ACE-inhibitory effect can be assessed by measuring tissue membrane-anchored or soluble, circulating ACE activities, and confirmed by measuring circulating levels of angiotensin II.[30] Our results show that serum ACE activity is reduced in SHRs after oral administration of both peptides. Moreover, inhibition of ACE was confirmed in peptide treated SHRs by the reduction in angiotensin II level. We have previously reported that long term administration to SHRs of an antihypertensive bovine LF pepsin hydrolyzate enriched in low molecular weight peptides reduced circulating ACE activity, angiotensin II and aldosterone levels.[10] By contrast, in the present study, the level of serum aldosterone, the adrenal endocrine component downstream angiotensin II in the renin-angiotensin axis,[2] was not affected by single-dose treatments with DPYKLRP and LRP. *In vivo* ACE inhibition has been also pointed out as the mechanism underlying the blood pressure reduction of the tripeptide IQP derived from the blue algae *Spirulina platensis* since serum ACE and angiotensin II levels were significantly reduced in SHRs after one-week treatment.[31] Nonetheless, the identification of other *in vivo* mechanisms beyond ACE inhibition underlying antihypertensive effects of the LF-derived peptides identified in this study should be further investigated.

Our results point out *K. marxianus* as a feasible GRAS microorganism for the production of novel LF-derived peptides with ACE-inhibitory and

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

ABBREVIATIONS USED

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

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513

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520

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

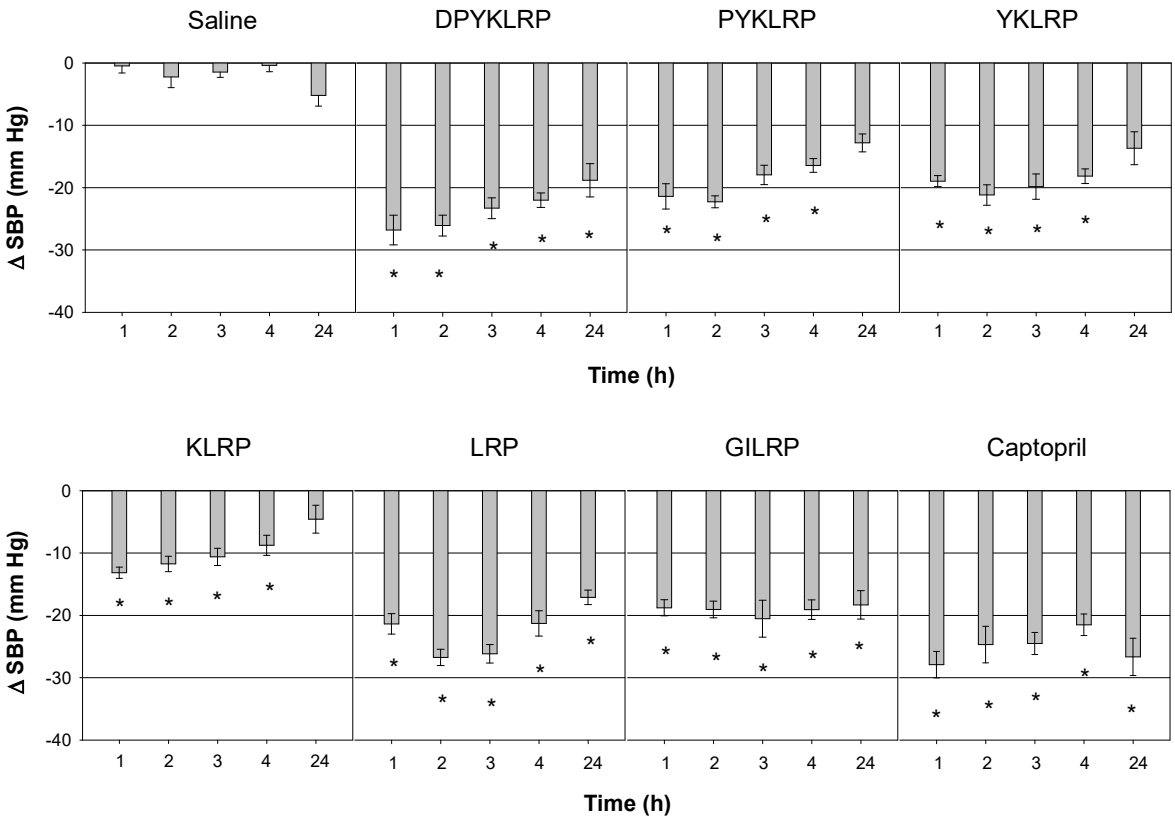
Table 2. Inhibitory Potency of Selected LF-Derived Peptides on ACE Activity

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).

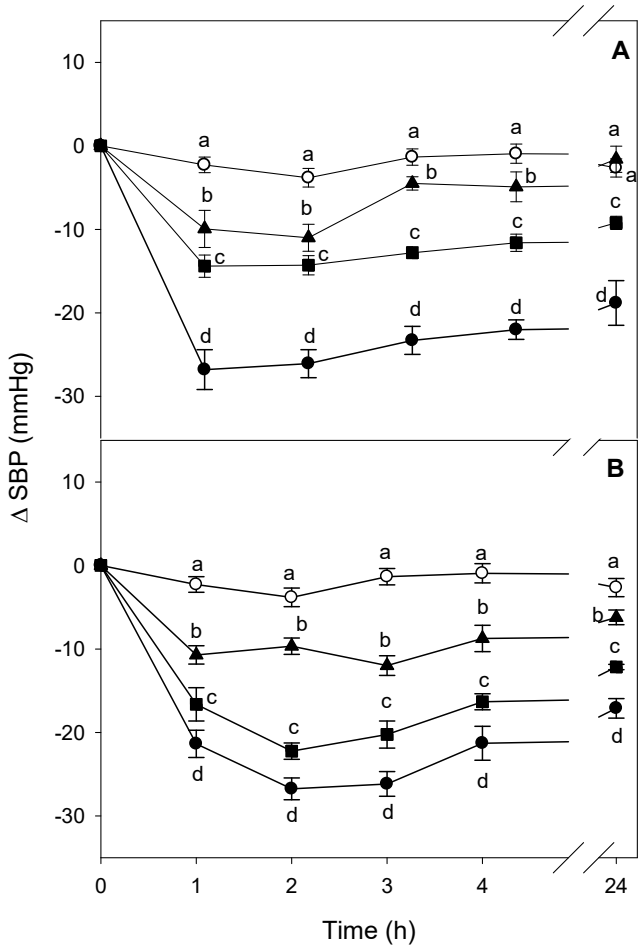
547

Figure 1


Saline
DPYKLRP
PYKLRP
YKLRP
KLRP
LRP
GILRP
Captopril
Δ SBP (mm Hg)
0
-10
-20
-30
-40
1
2
3
4
24
Time (h)

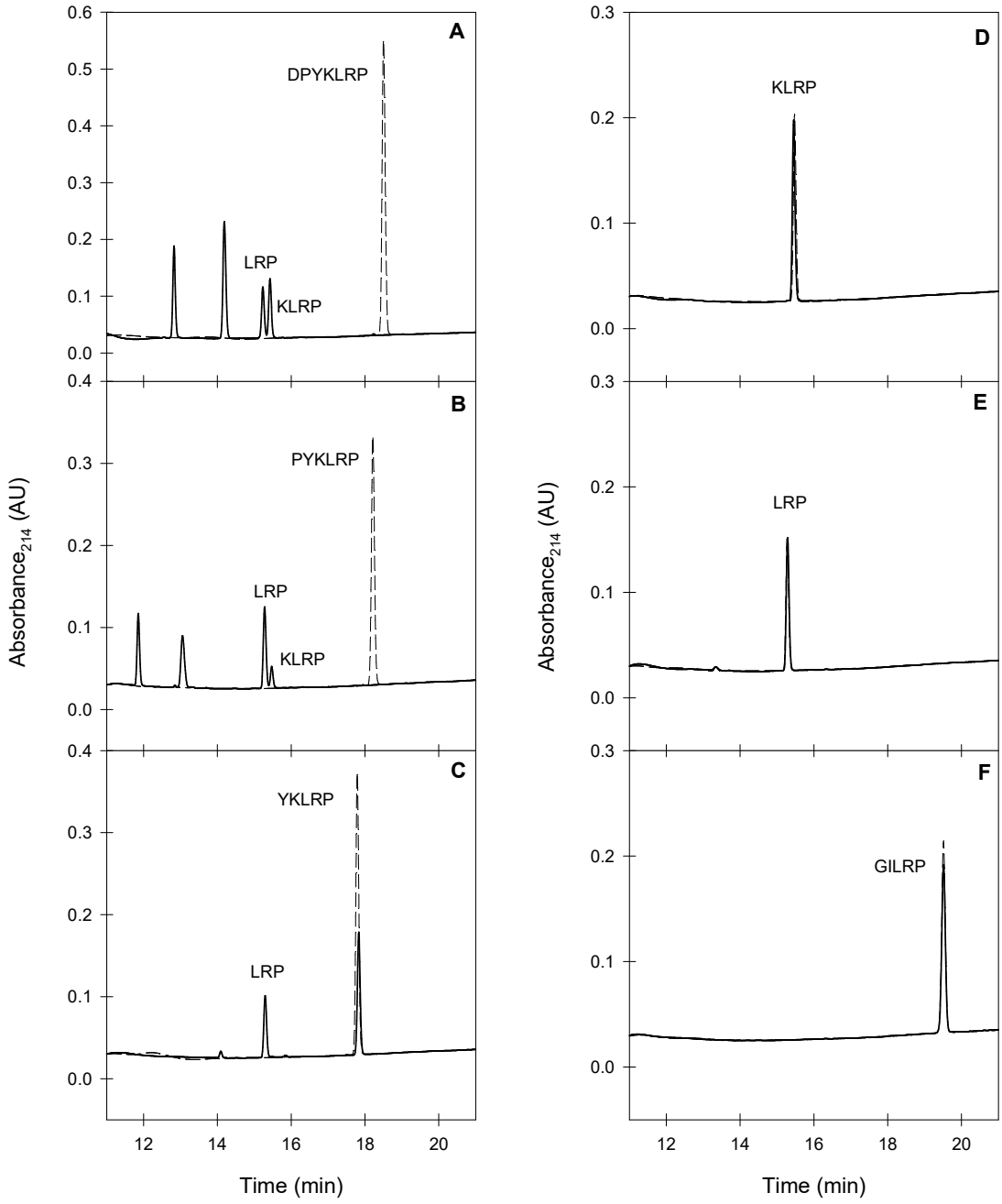
548

Figure 2



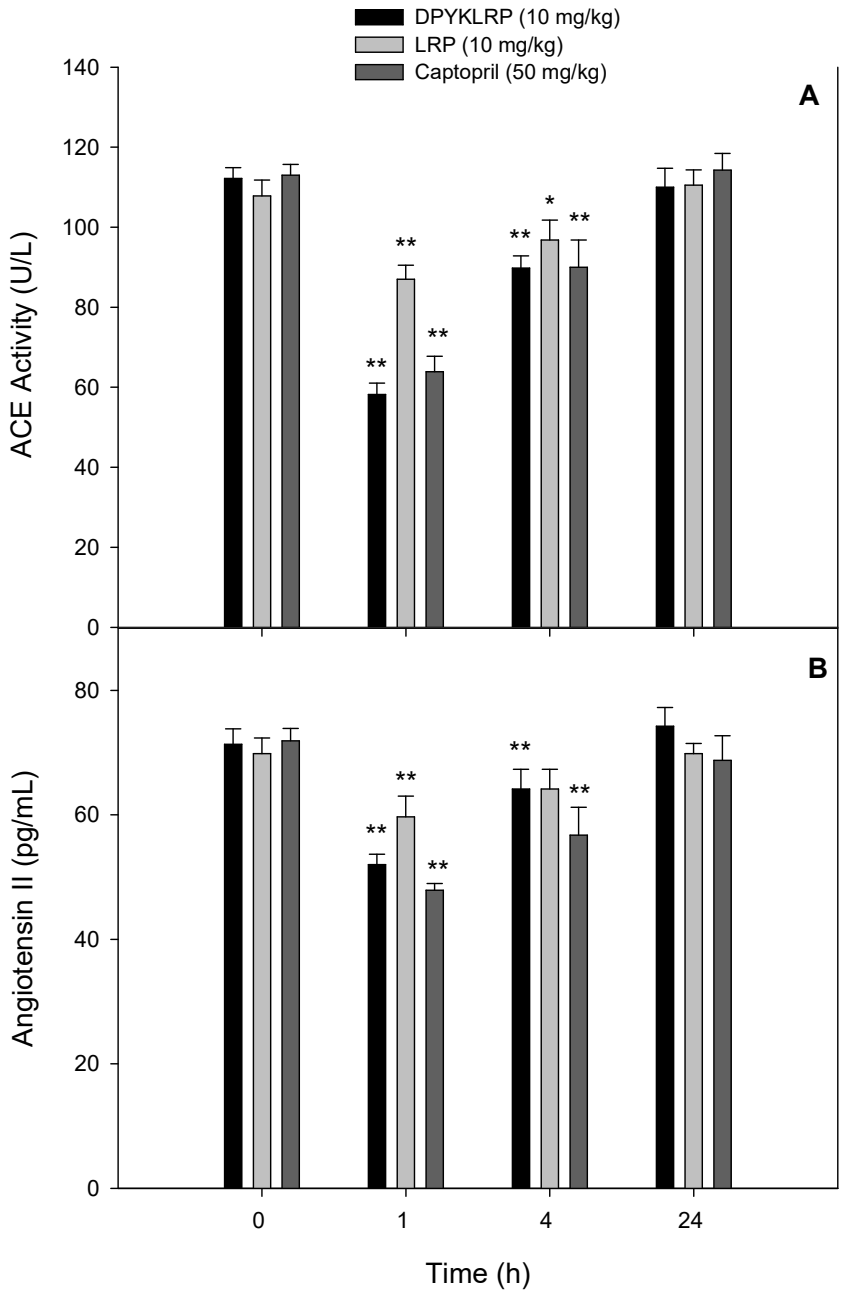
549

Figure 3


A
DPYKLRP
LRP
KLRP
B
PYKLRP
LRP
KLRP
C
YKLRP
LRP
D
KLRP
E
LRP
F
GILRP
Absorbance214 (AU)
Time (min)

550

Figure 4


DPYKLRP (10 mg/kg)
LRP (10 mg/kg)
Captopril (50 mg/kg)
A
B
ACE Activity (U/L)
Angiotensin II (pg/mL)
Time (h)
0
1
4
24
0
20
40
60
80
100
120
140
**
*

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