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Antihypertensive peptides from whey proteins fermented by lactic acid bacteria

Eric Banan-Mwine Daliri[1] · Byong H. Lee[1,2] · Byun-Jae Park[1] · Se-Hun Kim[1] · Deog-Hwan Oh[1]

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Abstract In this study, whey proteins were fermented with 34 lactic acid bacteria for 48 h at 37 °C and their ability to inhibit angiotensin 1-converting enzyme (ACE) activity were compared. All the lactic acid bacteria displayed varying proteolytic abilities in whey. Their fermentates also displayed varying abilities to inhibit ACE in vitro. Seven fermentates showed strong ACE inhibitory abilities between 84.70 ± 0.67 and 52.40 ± 2.1% with IC_{50} values between 19.78 ± 1.73 and 2.13 ± 0.7 mg/ml. *Pediococcus acidilactici* SDL1414 showed the strongest ACE inhibitory activity of 84.7 ± 0.67% (IC_{50} = 19.78 ± 1.73 µg/ml). Mass spectrometry revealed that more than half (57.7%) of the low molecular weight peptides (< 7 kDa) in the *P. acidilactici* SDL1414 fermented samples were ACE inhibitory peptides. Our results show that *P. acidilactici* SDL1414 could be used as a starter culture in the dairy industry to develop antihypertensive functional foods for hypertension management.

Keywords Fermentates · Angiotensin1-converting enzyme · Bioactive peptides

✉ Deog-Hwan Oh
deoghwa@kangwon.ac.kr

[1] Department of Food Science and Biotechnology, Kangwon National University, Chuncheon 200-701, South Korea

[2] Department of Food Science/Agricultural Chemistry, McGill University, Ste-Anne-de-Bellevue, QC H9X 3V9, Canada

Introduction

Food proteins usually contain several biologically active peptides. However, these peptides remain inactive as long as they remain bonded to other amino acids in the primary structure (Daliri et al., 2017a). Once released through enzymatic proteolysis and/or microbial fermentation, the free forms of the peptides demonstrate health effects in the gut or after systemic absorption into blood circulation. Several bioactive peptides have been reported to lower blood sugar, serum cholesterol, high blood pressure and inhibit microbial and cancer growth (Sánchez and Vázquez, 2017). Existing synthetic antihypertensive drugs have numerous side effects and this has increased scientific interest in searching for antihypertensive peptides as alternative therapeutics to control systemic blood pressure and to prevent cardiovascular diseases (Daliri et al., 2017b). One important enzyme involved in blood pressure regulation is angiotensin I-converting enzyme (ACE). The enzyme is a transmembrane metallopeptidase found in biological fluids and in many tissues such as the lung, thoracic aorta, heart, kidney, and liver (Nakamura et al., 2013). The enzyme hydrolyzes angiotensin I (a decapeptide) to yield angiotensin II (an octapeptide) which binds to AT1 receptors on vascular smooth muscles and endothelial cells leading to vasoconstriction. Angiotensin II also inactivates the endothelium-dependent vasodilator bradykinin (a nanopeptide) and this leads to high blood pressure (Manzanares et al., 2015). Therefore, inhibition of ACE activity is a good target for antihypertensive agents. Whey proteins such as beta-lactoglobulin, alpha-lactalbumin, bovine serum albumin, and immunoglobulin exhibit diverse physiological functions and their hydrolysates have been shown to have ACE inhibitory activities (Ibrahim et al., 2017). For instance the fragment 208–216 sequence

of serum albumin, fragments 102–105 and f146–149 sequences of β-lactoglobulin and also fragments 50–53 sequences of α-lactalbumin have ACE inhibitory properties (Ahn et al., 2009; Tavares et al., 2011). Many lactic acid bacteria (LAB) have been shown to degrade milk proteins to release bioactive peptides during diary fermentation. Such bacteria contain well developed proteolytic systems that break down proteins in the growth media into short amino acid chains (Daliri et al., 2017c). Given the proteolytic nature of LAB such as *Lactobacillus acidophilus*, *L. brevis*, *Lactobacillus animalis and Lactococcus lactis*, their use as microbial catalysts for producing ACE inhibitory peptides have been well documented (Brzozowski and Lewandowska, 2014; Hayes et al., 2007). *Lactobacillus helveticus* CP790 and *Saccharomyces cerevisiae* have been used as mixed cultures to produce potent ACE inhibitors such as Val-Pro-Pro and Ile-Pro-Pro from casein (Nakamura et al., 1995). Consuming milk containing the peptides effectively reduced high blood pressure in antihypertensive patients (Jauhiainen et al., 2010).

In this study we investigated the in vitro ACE inhibitory activity of 34 lactic acid bacteria during whey fermentation. Whey fermentates with the strongest ACE inhibition was analyzed by liquid chromatography-electrospray ionization-quantitative time-of-flight tandem mass spectrometry to identify the biopeptides that might have been involved in the inhibition.

Materials and methods

Chemicals and cultures

Unless specified, all chemical reagents were of analytical grade and were obtained from Sigma-Aldrich, South Korea. N-Hippuryl-His-Leu hydrate powder, ≥ 98% (HHL), ACE from rabbit lung, Phe-Gly, *O*-phthaldialdehyde (OPA), sodium tetraborate, sodium dodecyl sulfate, and whey from bovine milk (spray-dried) were purchased. Thirty-four lactic acid bacteria strains namely *Pediococcus pentosaceus* SDL1416, *Pediococcus acidilactici* SDL1405, *Enterococcus lactis* SCL1421, *Lactobacillus plantarum* JDFM44, *Weissella confusa* SCSB2320, *Lactobacillus rhamnosus* JDFM6, *Pediococcus acidilactici* SDL1414, *Enterococcus faecalis* MAD13, *Streptococcus thermophilus* SCML300, *Pediococcus pentosaceus* SDL1415, *Lactobacillus rhamnosus* JDFM33, *Streptococcus thermophilus* SCML337, *Pediococcus pentosaceus* SDL1409, *Pediococcus pentosaceus* SDL1401, *Pediococcus acidilactici* SDL1406, *Leuconostoc mesenteroides* JBNU10, *Leuconostoc citreum* SC53, *Pediococcus acidilactici* SKL1418, *Lactobacillus pentosus* SC48, *Enterococcus faecium* CK5, *Pediococcus pentosaceus* MAC11, *Leuconostoc paramesenteroides* SC46, *Weissella koreensis* JBNU2, *Lactobacillus curvatus* JBNU38, *Weissella confusa* SCKB2318, *Enterococcus faecium* SC54, *Lactobacillus brevis* SDL1411, *Lactobacillus brevis* SDL1408, *Pediococcus acidilactici* SCL1420, *Lactobacillus plantarum* SDL1413, *Weissella cibaria* SCCB2306, *Lactobacillus arizonensis* SC25, *Pediococcus acidilactici* DN9 and *Pediococcus acidilactici* SDL1402 were obtained from Soonchang Jang Ryu Saupso Company-Korea. Stock cultures were maintained at − 80 °C in de Man, Rogosa and Sharpe broth (MRS, MBCell-Korea), containing 20% (v/v) glycerol. Cultures were streaked on MRS agar and cultured at 37 °C for 24 h. Single colonies were then transferred into MRS broth at 37 °C and harvested at the exponential phase of growth. The viable bacterial count was determined by plate count on MRS agar. The basal growth medium for the lactic acid bacteria consisted of 2% (w/v) reconstituted whey powder in distilled water, supplemented with 1% glucose and 0.5% yeast extract (Becton–Dickinson Co., Cockeysville, MD). Whey powder was composed of 11% protein. The whey-based growth media were autoclaved at 121 °C for 15 min prior to inoculation with lactic acid bacteria.

Cultivation of lactic acid bacteria in whey media

To grow the lactic acid bacteria in whey-based growth media, 2×10^8 cfu/ml of each strain in the 24 h MRS broth at 37 °C were inoculated into 500 mL Erlenmeyer flask containing 200 ml of whey medium (pH 6). Cultivation was carried out at 37 °C with 150 rpm of agitation. After growth for 48 h, the media was centrifuged (10,000×*g*; 10 min) to remove precipitates. The supernatant (hydrolysate) was freeze-dried using TFD5505 table top freeze dryer (ilshinBioBase Co. Ltd, South Korea) and the freeze-dried samples were stored at − 80 °C for further analysis.

Determination of degree of protein hydrolysis

The degree of whey protein hydrolysis was carried out as reported earlier (Church et al., 1983). In brief, *O*-phthaldialdehyde (OPA) solution containing 40 mg of OPA dissolved in 1 ml of methanol, 100 ml of β-mercaptoethanol solution, 25 ml of 100 mM sodium tetraborate and 2.5 ml of 20% (w/w) sodium dodecyl sulfate (Sigma), was diluted to a final volume of 50 ml with water. The freeze-dried samples (5 mg/ml) were incubated with 1 ml OPA for 2 min at room temperature (∼ 28 °C) and absorbance at 340 nm was measured. The amount of peptide liberated during the fermentation process was calculated using Phe-Gly as a standard.

Recovery of low molecular weight peptides (< 7000 Da)

The hydrolysate (700 mg) was dissolved in 2 ml distilled water and dialyzed against water using a 7000 Da molecular weight cut-off dialysis bag (Viskase Corporation, Chicago, Illinois) for 48 h to recover low molecular weight peptides. We chose this molecular cut-off because most reported ACE inhibitory peptides are less than 10 kDa (Abdel-Hamid et al., 2017; Georgalaki et al., 2017). After discarding the retentates, the dialysates (permeates) were freeze-dried. The protein concentrations were determined using Bradford reagent according to the manufacturer's instructions.

In-vitro assay for ACE inhibitory activity

ACE inhibitory activity was measured by the procedures summarized in Table 1 (Cushman and Cheung, 1971). Briefly, 20 µl of the ACE inhibitor (LMW peptides) solution was mixed with 50 µl of 5 mM HHL in 100 mM sodium borate buffer (pH 8.3) containing 0.3 M NaCl and incubated at 37 °C for 5 min. The reaction was initiated by adding 10 µl of 0.1 U/ml ACE solution and the mixture was incubated at 37 °C for 30 min. The reaction was stopped by adding 100 µl of 1 M HCl and the reaction mixture was mixed with 1.0 ml of ethyl acetate. The mixture was vortexed for 60 s and centrifuged at 2000 × g for 5 min. An aliquot (800 µl) of ethyl acetate layer was transferred to a clean tube and evaporated on a water bath. Distilled water (800 µl) was then added to dissolve the hippuric acid (HA) remaining in the tube and the amount of HA formed was measured at 228 nm using a biospectrometer (Eppendorf Biospectrometer® fluorescence). The amount of HA liberated from HHL under this reaction conditions without an inhibitor was used as control. The extent of inhibition was calculated as $100\% \times [(B - A/B)]$ where A is the optical density (OD) in the presence of ACE and ACE inhibitory component, B is the OD without ACE inhibitor. For the determination of IC_{50}, series of dilutions containing 5000, 500, 50, 5, 0.5, and 0.05 µg/ml of the peptides were prepared. The amount of peptides required to suppress 50% ACE activity was calculated from the regression curves observed for each fraction.

Table 1 Procedure for assay of ACE inhibitory activity

	Blank/µL	Control/µL	Test/µL
ACE	10	0	0
HCL	100	0	0
HHL (substrate)	50	50	50
ACE inhibitor	20	0	20
Buffer	0	20	0
Incubation at 37° for 5 min			
ACE	0	10	10
Incubation at 37° for 30 min			
HCL		100	100
Ethyl acetate	1000	1000	1000

Identification of peptides by mass spectrometry

The peptide profile of the samples fermented with *P. acidilactici* SDL 1414 was determined using Liquid chromatography-electrospray ionization-quantitative time-of-flight tandem mass spectrometry experiments (LC-ESI-TOF–MS/MS). This fermentate was chosen for further analysis because it showed the lowest IC_{50} (highest potency). LC-ESI-TOF–MS/MS was performed at the National Instrumentation Center for Environmental Management of Seoul National University-Korea, using a method already described (Chang et al., 2014). Mass spectrometry was carried out using an integrated system comprising an autosampler (TempoTM nano LC system; MDS SCIEX, Canada), an auto-switching nano pumpand a hybrid quadrupole-time-of-flight (TOF) mass spectrometer (QStar Elite; Applied Biosystems, USA) with a fused silica emitter tip (New Objective, USA). Nano-electrospray ionization (ESI) was applied for sample ionization. The sample (2 µl) was injected into the LC-nano ESI–MS/MS system. The sample was initially trapped on a ZORBAX 300SB-C18 trap column (300-µm i.d × 5 mm, 5-µm particle size, 100 pore size, Agilent Technologies, part number 5065-9913) and washed for 6 min with gradient with 98% solvent A and 2% solvent B at a flow rate of 5 µL/min. Solvents A and B consisted of [water/acetonitrile (98:2, v/v), 0.1% formic acid] and [Water/acetonitrile (2:98, v/v), 0.1% formic acid] respectively. Separation was carried out using a ZORBAX 300SB-C18 capillary column (75-µm i.d × 150 mm, 3.5 µm particle size, 100 pore size, part number 5065-9911) at a flow rate of 300 nl/min with gradient at 2 to 35% solvent B over 30 min, then from 35 to 90% over 10 min, followed by 90% solvent B for 5 min, and finally 5% solvent B for 15 min. Electrospray was performed through a coated silica tip (FS360-20-10-N20-C12, PicoTip emitter, New Objective) at an ion spray voltage of 2000 eV. Using Analyst QS 2.0 software (Applied Biosystems, USA), the peptides were analyzed automatically. The range of m/z values was 200-2000.

Statistical analysis

All experiments were carried out in triplicates and the results were expressed as the mean ± standard deviation.

The statistical analysis of data was performed using GraphPad Prism 5.0 (2007) statistical software system (GraphPad Software Inc. CA 92037 USA). $P < 0.05$ was considered significant.

Results and discussion

Degree of whey hydrolysis by lactic acid bacteria

The degree of whey protein hydrolysis by each of the 34 LAB was tested after fermentation for 48 h. LAB require exogenous amino acids for growth and they derive these amino acids from the protein substrates in which they are cultured. They do this by hydrolyzing proteins into oligopeptides using their cell-enveloped proteinase (Liu et al., 2010). Specific peptide transport systems then transport the oligopeptides into the cells where they are hydrolyzed into amino acids by various intracellular peptidases (Daliri et al., 2017c). All the LAB in this study showed varying abilities to hydrolyze whey (Fig. 1) and this agrees with earlier reports that showed that LAB protein digestion is strain dependent (Bounouala et al. 2017). We observed that *L. plantarum* JDFM44 showed the highest ability to hydrolyze whey proteins followed by *L. plantarum* SDL1413 (Fig. 1). This could be due to the release of large amounts of exopeptidases which hydrolyzed N-terminal amino acids from the proteins during the fermentation process. *Ent. faecium* CK-5, *P. pentosaceus* SDL1401, *L. pentosus* SC48 and *P. acidilactici* SDL1414 also showed > 50% degree of hydrolysis while the remaining strains showed low degrees of hydrolysis during the fermentation process.

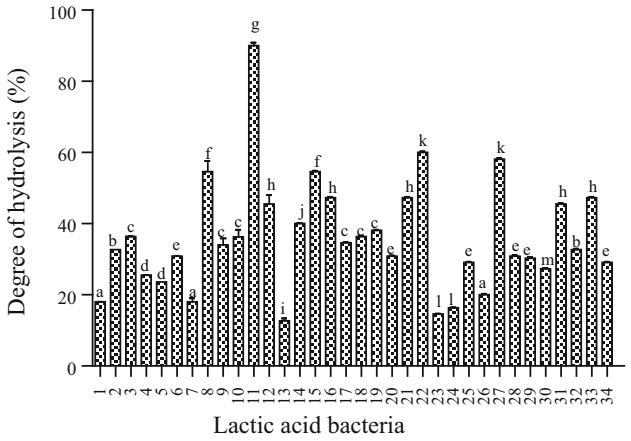

Fig. 1 The degree of whey hydrolysis by 34 lactic acid bacteria after 48 h fermentation at 37 °C. Degree of hydrolysis is mean ± SEM of three independent experiments. Bars with different alphabets are significantly different ($p < 0.05$). Legend: 1—*L. rhamnosus* JDFM6; 2—*P. Pentosaceus* SDL1415; 3—*P. Pentosaceus* SDL1416; 4—*L. rhamnosus* JDFM33; 5—*E. lactis* SCL1421; 6—*P. acidilacti* SDL1405; 7—*W. confusa* SCSB2320; 8—*P. acidilactici* SDL1414; 9—*S. thermophillus* SCML300; 10—*Ent. Faecalis* MAD13; 11—*L. planterum* JDFM44; 12—*Ent. fecium* SC54; 13—*P. acidilactici* SCL1420; 14—*W. koreensis* JBNU2; 15—*L. pentosus* SC48; 16—*Leu. paramesenteroides* SC46; 17—*P. acidilactici* SKL1418; 18—*L. brevis* SDL1408; 19—*L. brevis* SDL1411; 20—*W. confusa* SCKB2318; 21—*L. curvatus* JBNU38; 22—*Ent. fecium* CK5; 23—*S. thermophilus* SCML337; 24—*L. plantarum* SDL1413; 25—*L. arizonensis* SC27; 26—*L. pentosaceus* SDL1409; 27—*L. pentosus* SDL1401; 28—*P. acidilactici* SDL1406; 29—*Leu. mesenteroides* JBNU10; 30—*W. cibaria* SCCB2306; 31—*P. pentosaceus* SDL1416; 32—*P. acidilactici* DM9; 33—*P. pentosaceus* MAC11; 34—*L. citrium* SC53

Determination of in vitro ACE inhibitory (ACEI) activity in low molecular weight (LMW) peptides

All the fermentates showed varying ACEI ability (data not shown) however, the fermentates that had ≥ 50% is shown in Table 2 and their IC_{50} were determined. When LMW peptides (< 7 kDa) were recovered from these samples, 17 fermentates had ACEI ability (data not shown). LMW peptides from *P. acidilactici* SDL1414 fermentation showed the strongest in vitro ACEI activity of 84.7 ± 0.67 with an IC_{50} value of 19.78 ± 1.73 μg/ml (Table 2). Captopril (a synthetic ACEI compound) showed an inhibition of 90.82 ± 10.3 and an IC_{50} value of 5 ± 0.1 μg/ml. Interestingly, we did not find any correlation between the degree of hydrolysis and ACE inhibitory potency. Since LMW peptides from *P. acidilactici* SDL 1414 fermentation showed the highest ACE inhibition, they were chosen for further studies using LC–ESI–MS/MS to identify the antihypertensive peptides generated during the fermentation process.

Table 2 ACE-inhibitory activities in the low-molecular-weight fraction (< 7 kDa) of whey fermentates by lactic acid bacteria

Samples	ACEI (%)	[a]IC_{50} (μg/mL)
P. acidilactici SDL1414	84.70 ± 0.67	19.78 ± 1.73
L. plantarum JDFM44	84.00 ± 1.05	65.53 ± 7.99
Ent. faecium SC54	55.40 ± 1.73	70.50 ± 11.2
P. acidilactici DM9	54.90 ± 0.90	96.70 ± 20.86
L. berevis SDL1411	79.03 ± 3.40	1280 ± 300
P. pentosaceus SDL1409	72.90 ± 1.60	2070 ± 550
L. rhamnosus JDFM6	52.40 ± 2.10	2130 ± 700
Captopril (control)	90.82 ± 10.30	5.0 ± 0.10

[a]Concentration of peptide needed to inhibit 50% of original ACE activity. The data in this table represent samples that showed ACEI ability ≥ 50% out of 34 fermented samples

Table 3 Whey-derived peptides identified by LC-ESI-TOF–MS/MS in the LMW peptides (< 7 kDa) fraction of the 48 h whey fermentates of whey by *P. acidilactici* SDL1414

Protein name	Peptide sequence	Prec MW	Prec m/z	Theor MW	Theor m/z	z
α_{S1}-CN	(f10–23) GLPQEVLNENLLRF	1640.9009	821.4577	1640.8885	821.4516	2
	(f10–22) GLPQEVLNENLLR	1493.8313	747.9229	1493.8202	747.9174	2
	(f1–23) RPKHPIKHQGLPQEVLNENLLRF	3281.7998	821.4572	3286.8779	822.7267	4
	(f14-23) EVLNENLLRF	1245.6793	623.8469	1245.6718	623.8431	2
	(f10-21) GLPQEVLNENLL	1337.7279	669.8712	1337.7191	669.8668	2
	(f24-34) FVAPFPEVFGK	1236.6609	619.3377	1236.6543	619.3344	2
	(f25-34) VAPFPEVFGK	1089.5898	545.8022	1089.5859	545.8002	2
	(f24-38) FVAPFPEVFGKEKVNEL	1949.0464	650.6894	1949.0299	650.6839	3
	(f80-98) HIQKEDVPSERYLGYLEQL	2316.1978	580.0567	2316.175	580.051	4
β-CN	(f1-27) RELEELNVPGEIVESL	1823.9845	912.9995	1824.9469	913.4807	2
	(f1-25) RELEELNVPGEIVE	1623.8574	812.936	1624.8308	813.4227	2
	(f1-22) RELEELNVPGE	1283.6456	642.8301	1283.6357	642.8251	2
	(f1-24) RELEELNVPGEIV	1494.8132	748.4139	1494.8042	748.4094	2
	(f192-209) LYQEPVLGPVRGPFPIIV	1993.1543	665.392	1993.14	665.3873	3
	(f193-209) YQEPVLGPVRGPFPIIV	1880.0715	941.043	1880.056	941.0353	2
	(f193-208) YQEPVLGPVRGPFPII	1781.0021	891.5083	1780.9875	891.5011	2
	(f194-209) QEPVLGPVRGPFPIIV	1699.9836	850.9991	1699.9662	850.9904	2
	(f195-209) EPVLGPVRGPFPIIV	1588.9463	795.4804	1588.9341	795.4743	2
	(f83-95) VVPPFLQPEVMGV	1410.7693	706.3919	1410.7581	706.3864	2
κ-CN	(f161-169) TVQVTSTAV	904.49048	453.2525	904.48657	453.2505	2
	(f155-169) SPPEINTVQVTSTAV	1541.8065	771.9105	1541.7937	771.9041	2
	(f149-169) SPEVIESPPEINTVQVTSTAV	2276.0999	759.7072	2276.0825	759.7014	3
	(f151-169) EVIESPPEINTVQVTSTAV	2012.0497	671.6905	2012.0314	671.6844	3
	(f159-169) INTVQVTSTAV	1131.6196	566.8171	1131.6135	566.814	2
	(f152-169) VIESPPEINTVQVTSTAV	1883.0045	942.5095	1882.9888	942.5016	2
	(f150-169) PEVIESPPEINTVQVTSTAV	2109.0984	704.0401	2109.0842	704.0353	3
	(f157-169) PEINTVQVTSTAV	1357.7208	679.8677	1357.7089	679.8618	2
	(f151-169) EVIESPPEINTVQVTSTAV	2034.0271	679.0163	2034.0133	679.0117	3
	(f151-165) EVIESPPEINTVQVT	1653.8649	827.9397	1653.8462	827.9304	2
	(f151-163) EVIESPPEINTVQ	1453.7411	727.8778	1453.73	727.8723	2
	(f149-162) SPEVIESPPEIN	1309.6481	655.8313	1309.6401	655.8273	2
	(f149-163) SPEVIESPPEINTVQ	1717.8009	859.9077	1717.781	859.89	2
	(f151-162) EVIESPPEIN	1125.5629	563.7887	1125.5554	563.785	2
	(f116-141) MAIPPKKNQDKTEIPTINTIASGEPT	2793.4849	699.3785	2793.4583	699.3718	4
	(f109-151)PPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDSPE	4523.3027	1131.833	4523.299	1131.81	4
	(f106-149)MAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLED	4525.3066	1132.334	4525.263	1132.32	4

Table 3 continued

Protein name	Peptide sequence	Prec MW	Prec m/z	Theor MW	Theor m/z	z
β-lg	(f130-149) DEALEKFDKALKALPMHIRL	2353.2981	471.6669	2353.2827	471.6638	5
	(f130-146) DEALEKFDKALKALPMH	1955.031	489.765	1955.0187	489.7619	4
	(f130-145) DEALEKFDKALKALPM	1817.973	606.9983	1817.9597	606.9938	3
	(f1-11) LIVTQTMKGLD	1217.6791	609.8468	1217.6689	609.8417	2
	(153-162) PTQLEEQCHI	1162.5697	582.2921	1162.5619	582.2882	2
	(147-156) IRLSFNPTQL	1187.6759	594.8452	1187.6663	594.8404	2
	(f1-11) LIVTQTMKGL	1102.6472	552.3309	1102.642	552.3283	2
	(f1-12) LIVTQTMKGLD	1217.6791	609.8468	1217.669	609.842	2
	(f153-162) PTQLEEQCHI	1162.5697	582.2921	1162.562	582.288	2
	(f147-156) IRLSFNPTQL	1187.6759	594.8452	1187.666	594.84	2
	(f1-10) LIVTQTMKGL	1102.6472	552.3309	1102.624	552.328	2
Lactophorin (PP3)	(f1-18) ILNKPEDETHLEAQPTDA	2019.999	674.3403	2019.9749	674.3323	3
	(f1-17) ILNKPEDETHLEAQPTD	1948.9561	650.6593	1948.9377	650.6532	3
	(f57-67) QPQSQNPKLPL	1231.6656	616.8401	1231.6561	616.8353	2
	(f54-67) SSRQPQSQNPKLPL	1578.8573	527.293	1578.8478	527.2899	3
PIGR	(f383-404) PGRPTGYSGSSKALVSTLVPLA	2157.1958	720.0726	2157.1794	720.0671	3
UP (GP2)	(f455-473) SEGVAIDPARVLDLGPITR	1978.1027	660.3748	1978.0847	660.3688	3

CN casein, *m/z* mass to charge ratio, where *z* number of positively charged ions, *Prec* precision, *Theor* theoretical

α_{S1}*-CN* alpha-S1-casein, *κ-CN* kappa-casein, *β-CN* beta-casein, *β-lg* beta lactoglobulin, *PP3* proteose-peptone component 3, *UP (GP2)* uncharacterized protein GP2; *PIGR* polymeric immunoglobulin receptor

Table 4 Antihypertensive and potential antihypertensive peptides generated by whey fermentation with *P. acidilactici* SDL1414 after 48 h at 37 °C compared with ACEI peptides reported in literature

Name	ACEI peptides reported in literature	Present study[a]	References
α_{S1}-CN	(f14-23) EVLNENLLRF	(f14-23) **EVLNENLLRF**[b]	Torres-Llanez et al. (2011)
		(f10–23) GLPQ**EVLNENLLRF**	
		(f10–22) GLPQ**EVLNENLLR**	
		(f10-21) GLPQ**EVLNENLL**	
	(f23-34) FFVAPFPEVFGK	(f24-38) **FVAPFPEVFGK**EKVNEL	Hayes et al. (2007)
		(f24-34) **FVAPFPEVFGK**	
		(f25-34) **VAPFPEVFGK**	
β-CN	(f7-16) NVPGEIVESL	(f1-27) RELEEL**NVPGEIVESL**	Hayes et al. (2007) and Jin et al. (2016)
	(f6-14) LNVPGEIVE	(f1-25) RELEE**LNVPGEIVE**	
	(f2-11) ELEELNVPGE	(f1-22) R**ELEELNVPGE**	
		(f1-24) RELE**ELNVPGEIV**	
	(f74-97) NIPPLTQTPVVVPPFIQPEVMGVSK	β-CN (f83-95) **VVPPFLQPEVMGV**	Hayes et al. (2007)
	(f191-209) LLYQEPVLGPVRGPFPIIV	(f192-209) **LYQEPVLGPVRGPFPIIV**	Jin et al. (2016)
		(f193-209) **YQEPVLGPVRGPFPIIV**	
		(f193-208) **YQEPVLGPVRGPFPII**	
		(f194-209) **QEPVLGPVRGPFPIIV**	
		(f195-209) **EPVLGPVRGPFPIIV**	
κ-CN	(f161-169) TVQVTSTAV	(f161-169) **TVQVTSTAV**	Tavares et al. (2011)
	(f64-69) VTSTAV	(f155-169) SPPEINTVQ**VTSTAV**	
		(f149-169) SPEVIESPPEINTVQ**VTSTAV**	
		(f151-169) EVIESPPEINTVQ**VTSTAV**	
		(f159-169) INTVQ**VTSTAV**	
		(f152-169) VIESPPEINTVQ**VTSTAV**	
		(f150-169) PEVIESPPEINTVQ**VTSTAV**	
		(f157-169) PEINTVQ**VTSTAV**	
		(f151-169) EVIESPPEINTVQ**VTSTAV**	
β-lg	(f142-148) ALPMHIR	(f130-149) DEALEKFDKALK**ALPMHIR**L	Tavares et al. (2011) and Nagpal et al. (2011)
		(f130-146) DEALEKFDKALK**ALPMH**	
		(f130-145) DEALEKFDKALK**ALPM**	

[a]Peptides obtained from whey fermentation in the present study. Segments homologous to previously identified ACEI peptides are in bold letters

[b]Peptide identified in this study which is identical to a previously identified ACEI peptide

Peptides generated from whey during fermentation by *Pediococcus acidilactici* SDL1414

LC-ESI-TOF–MS/MS analysis of the LMW peptide profile showed that the peptides were generated from casein, beta lactoglobulin, lactophorin, polymeric immunoglobulin receptor and uncharacterized protein GP2 (Table 3). This indicates that *P. acidilactici* SDL1414 has the ability to hydrolyze different substrates present in the media relative to other lactic acid bacteria such as *Bifidobacterium longum* which preferentially hydrolyze only casein (Ha et al., 2015). During the fermentation process, a large number of peptides were generated from the C-terminus of β-CN but the N-terminal was resistant to hydrolysis probably due to the presence of several phosphoserine residues at that region (Kaspari et al., 1996). The resistance of the N-terminal region of β-CN to LAB hydrolysis has also been reported in earlier studies (Ha et al., 2015). Hydrolysis of the C-terminal region on the other hand could be due to its hydrophobic nature which makes it more accessible for hydrolysis (Chang et al., 2014). Other LAB including *Lactobacillus helveticus* (Griffiths and Tellez, 2013), *Lactobacillus delbrueckii* subsp. *lactis* lactis (Tsakalidou et al., 1999), *S. thermophilus* (Miclo et al.,

2012), and *Lactobacillus lactis* (Juillard et al., 1995) also readily hydrolyze the C-terminal region of β-CN to generate large numbers of peptides during milk fermentation.

The N terminus of α_{S1}-CN was also found to be resistant to *P. acidilactici* SDL1414 hydrolysis and this could also be due to the presence of phosphoserine residues in that region (Ha et al., 2015). The bacterium however readily hydrolyzed the C-terminus of the protein during the fermentation process.

The caseinomacropeptide or glycomacropeptide (f 106-169) segment of κ-CN is composed of glycan chains and has been reported to be resistant to hydrolysis in many studies (Svanborg et al., 2016). The resistance has been attributed to the presence of hydrophilic amino acids and their negative charges which cause increased electrostatic repulsion (Zahraa et al., 2010). However, in the present study, 17 different peptides were obtained from the region (Table 3), 9 of which have bear sequences already reported to have ACE inhibition (Table 4). *P. acidilactici* SDL1414 fermentation might have induced structural changes in the glycomacropeptide region to allow hydrolysis.

Many ACEI peptides have been reported to be present in whey. LC-ESI-TOF–MS/MS analysis of our low molecular weight peptides showed the presence of 52 different peptides (Table 3). Among these peptides, we identified 1 peptide, α_{S1}-CN (f14-23) EVLNENLLRF, which has already been reported to be an ACE inhibitor (Torres-Llanez et al., 2011). Also, 28 peptides generated in this study had their C-terminal amino acid sequences homologous with previously identified ACEI peptides (Table 4), yet, the complete sequences observed in this study have not been reported before (Kumar et al., 2014; Minkiewicz et al., 2008).

The abundance of antihypertensive segments in these peptides may at least, in part, account for the strong ACE inhibitory activity of the fermentate and the low IC_{50} (19.78 ± 1.73 μg/ml) observed in this study. Though our ACEI peptides are less potent than captopril (IC_{50} = 5 μg/ml), natural antihypertensive peptides have high tissue affinities and hence may be more slowly eliminated from tissues compared to synthetic drugs (Koyama et al., 2014). The slow elimination could enhance a more sustained antihypertensive effect in consumers. There is therefore a demand for such bioactive functional ingredients and that warrants this type of research.

The current study shows that *P. acidilactici* SDL1414 could be used as a starter culture in the dairy industry to develop antihypertensive functional foods. The peptides in the fermentates could also be purified and used as nutraceuticals or supplements to reduce high blood pressure. However, in vivo studies regarding the stability of these peptides against gastrointestinal enzymes as well as their blood pressure lowering ability is warranted.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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